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THE EFFECT OF PYOCYANIN ON HUMAN SKIN CELLS AND LEUCOCYTES

C. N. D. CRUICKSHANK AND E. J. L. LOWBURY

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Received for publication June 18, 1953.

THE dressings of burns infected with *Pseudomonas pyocyanea* often have a blue-green colour, which is due to the presence of pyocyanin and other pigments in the exudate. There is evidence that *Ps. pyocyanea* may cause the failure of skin grafts on burns and delay healing (Jackson, Lowbury and Topley, 1951). To find whether pyocyanin may play a part in causing these adverse results, we have studied the effect of different concentrations of the compound on human epithelial cells and leucocytes, and have attempted to assess the concentration of pyocyanin in green exudate from burns. These studies and a few animal experiments to determine the toxic rôle of pyocyanin in infection with *Ps. pyocyanea* are described in this paper.

Effect of Pyocyanin on Tissue Cultures of Human Skin.

Materials and methods.

The method of tissue culture, a modification of one described by Medawar (1948), was previously used by the authors in studying the effect of antibiotics on human skin (Cruickshank and Lowbury, 1952).

Solutions of pyocyanin (pyocyanine dihydrochloride, supplied by S. A. F. Hoffmann-La Roche & Co., Ltd.) were made in Ringer's solution at concentrations of 24·0, 2·4, 0·8, 0·24, 0·08, 0·024 and 0·008 mg. per ml. The culture medium consisted of 5 parts fresh homologous serum, 2 parts Krebs-Ringer phosphate, 1 part 5 per cent glucose in distilled water, 1 part pyocyanin solution and 1 part dihydrostreptomycin solution (500 units/ml. in Ringer's solution) to prevent the growth of contaminants. In the control medium the pyocyanin was omitted and the amount of Krebs-Ringer phosphate was increased to 3 parts. The medium had a pH of 7·2–7·4.

The excess of skin removed for storage at grafting operations was obtained, a specimen of medium thickness cut on the same day being used in each experiment. It was divided into pieces of about 1–2 sq. mm., which were floated on the surface of 0·5 ml. of culture medium in 5 ml. bottles with rubber stoppers, 3 bottles of each test dilution under study and of the control being set up at each experiment. The cultures were incubated at 37° in an atmosphere of oxygen, being rocked through 30 degrees about 5 times a min. on a platform.

After 3 days' incubation, the explants were fixed in formol saline, dehydrated, cleared and embedded in paraffin. Median sections were cut and stained with haematoxylin and eosin.

Results.

The appearance of sections is shown in Fig. 1–3. In the control culture epithelium has migrated from the edge of the explant to cover the cut surface of the dermis, which becomes encysted with new epithelium two or three cells thick. The dermis has the same appearance as that of newly cut skin. Two degrees of damage to the skin culture are described: (1) *Inhibition*: In these cultures the cells of the epidermis are still histologically normal but the amount of migration is less than in the controls. (2) *Destruction*: In this group there is no migration of epithelium, and the staining properties of cells are abnormal. The cultures are on occasion completely necrotic.

The effects of pyocyanin are shown in Table I. It will be noted that the highest concentration at which consistently good cultures were obtained was 0·0024 mg./ml. At concentrations from 0·008 to 0·24 mg./ml. the results varied from minor inhibition to complete destruction; at concentrations above 0·024 mg./ml. death of the explant was usual.

TABLE I.—*Effect of Pyocyanin on Cultures of Human Skin.*

Dilution of pyocyanin.	Number of explants showing			Ratio: normal/ abnormal.
	Normal growth.	Inhibition.	Necrosis.	
0·24 mg./ml.	—	—	9	0/9
0·08 ,,	—	—	3	0/3
0·024 ,,	—	2	9	0/11
0·008 ,,	5	4	—	5/4
0.0024 ,,	12	—	—	12/0
Controls	15	—	—	15/0

Effect of Pyocyanin on Human Leucocytes.

Materials and Methods.

The technique used by Abraham, Chain, Fletcher, Gardner, Heatley, Jennings and Florey (1941) for determining the toxic action of penicillin on leucocytes was adopted.

Drops of blood were allowed to clot at 37° on cover slips in a Petri dish containing moist filter paper. After about 30 min. the Petri dish was taken from the incubator and the blood clots were removed from the cover slips with forceps. Erythrocytes were washed away with warm Ringer's solution, and the cover slips were inverted over well-slides containing dilutions of pyocyanin in a special salt solution consisting of urea 0·3 g., glucose 1·0 g., $NaHCO_3$ 1·61 g., $NaH_2PO_4H_2O$ 0·425 g., NaCl 4·95 g., KCl 0·625 g., $MgCl_2$ 0·24 g., $CaCl_2$ 0·31 g., in 1 l. distilled water, with 10 per cent fresh human serum added at the time of the experiment. The dilutions of pyocyanin tested were 0·24, 0·08 and 0·024 mg./ml., 3 cover slips being prepared for each dilution and for controls without pyocyanin. The cover slips were sealed with paraffin wax.

Observations were made at hourly intervals for 4 hr. These were carried out with the microscope in an incubator box at 37° using an oil immersion objective. While not under observation the slides were kept at 37°. The motility and cytoplasmic movements of the granulocytes in a large number of fields were noted. Three experiments were made on different occasions.

Results.

When leucocytes were maintained in the presence of the "control" solution cytoplasmic movement remained vigorous for 4 hr. In pyocyanin 0·08 mg./ml.

and 0·024 mg./ml. the movement after 4 hr. was not impaired. In 0·2 mg./ml., however, the number of leucocytes active after 1 hr. was obviously reduced, and at 4 hr. all the cells appeared to be dead.

The Concentration of Pyocyanin in Exudate of Burns.

An approximate measure of the concentration of pyocyanin was obtained by extracting with a measured volume of chloroform a piece of gauze containing a known weight of exudate soaked up from a burn. The red colour reaction (acid pyocyanin) obtained on extracting the chloroform solution with acid was matched against a standard, and from the concentration of pyocyanin thus estimated to be present in the chloroform extract it was possible to assess the approximate concentration of pyocyanin in the exudate.

Method.

Absorbent gauze pads, 8 in. by 4 in. (20 × 10 cm.), were weighed, marked with black thread, wrapped in paper and sterilised by dry heat for 1 hr. at 160°.

Patients whose burns were colonised with *Ps. pyocyanea* and covered with green-stained dressings were selected for the investigation. Weighed gauze pads were included in the dressing under the cotton wool. The pads were removed when dressings were next changed (usually on the 3rd or 4th day), and if they were soaked with green exudate they were collected in glass ointment jars with screw-caps and re-weighed.

For the extraction and estimation of pyocyanin, the gauze pad was cut into small squares and returned to the ointment jar. A measured volume (50 ml.) of chloroform was then added, and the pieces of gauze were thoroughly extracted by stirring with a thick glass rod and shaking. Supernatant fluid was removed and centrifuged in small screw-cap bottles. It was then shaken with an equal volume of 0·1 N HCl, which extracted the pyocyanin as a red pigment (acid pyocyanin), the colour of which was matched against a range of doubling dilutions of acidified pyocyanin.

Results.

The approximate concentration of pyocyanin in the exudates from 5 burns (Table II) varied with the staining of the gauze and reached 0·31 mg./g. when

TABLE II.—*Approximate Concentration of Pyocyanin in Exudate from Five Burns.*

Patient.	Appearance of dressing.	Pyocyanin concentration (mg./g.)
1	Diffusely pea-green	0·07
2	Green and greenish yellow over part of gauze	0·008
3	Blue-green over most of gauze	0·22
4	Ditto	0·31
5	Patches of deep and pale green over most of gauze	0·14

the colour was bluish green, which is common in burns yielding heavy growths of *Ps. pyocyanea*.

These concentrations were similar to those obtained from 8-day agar slope cultures by extraction with chloroform (0·037 to 0·15 mg./ml.).

The Effects of Intradermal Inoculation of Guinea-pigs with Ps. Pyocyanea *and Pyocyanin.*

Fox and Lowbury (1953) have shown a difference between the size of small lesions produced on intradermal injection of rabbits with a strain of *Ps. pyocyanea* against which the animal had been immunised, and the larger lesions produced at the site of inoculation of unrelated strains of *Ps. pyocyanea*. By comparing the sizes of the lesions produced by strains of *Ps. pyocyanea* which did and which did not produce pyocyanin, it was hoped to obtain evidence of the rôle of pyocyanin in deeper infections. To the same end a series of intradermal inoculations of pyocyanin in Ringer's solution was made, and the effects observed.

Method.

Twelve strains of *Ps. pyocyanea* were used, of which 6 produced abundant pyocyanin and 6 produced no pyocyanin after 42 hr. culture on nutrient agar plates (18 hr. at 37° and 24 hr. on the bench); presence or absence of pyocyanin was confirmed by chloroform extraction of the fluid obtained after freezing and thawing the agar culture.

Three (in one case 2) intradermal injections in epilated guinea-pigs were made with undiluted and 10^{-1} dilutions of suspensions of each strain made by re-suspending washed broth culture in an equal volume of Ringer's solution. The red lesions, many of which had a necrotic and haemorrhagic centre, were examined and 2 diameters were measured with dividers after 24 and 48 hr.

The guinea-pigs were killed after 48 hr. and 1 lesion produced by each of the strains was excised, cut into small pieces, and extracted with chloroform to detect the presence of pyocyanin.

Four guinea-pigs were each given intradermal inoculations of 4 tenfold dilutions of pyocyanin in Ringer's solution (2·4, 0·24, 0·024 and 0·0024 mg./ml.). The guinea-pigs were examined after 24 hr., and lesions were excised for histological study.

Results.

There was no consistent difference in the size of lesions from the strains that produced pyocyanin and from those that did not produce the pigment.

Chloroform extracts of all lesions remained colourless: no evidence was therefore obtained of production of pyocyanin in the lesion.

The guinea-pigs given intradermal inoculations of pyocyanin showed slight macroscopic oedema, but negligible histological changes; some infiltration with round cells was found at the sites of inoculation of the most concentrated solution but most of the other inoculation sites appeared normal.

DISCUSSION.

These experiments show that pyocyanin is toxic to human epidermis cultivated *in vitro*, at concentrations above 0·0024 mg./ml. Schoental (1941) demonstrated that pyocyanin inhibited the growth of chick embryo fibroblasts at concentrations above 0·025 mg./ml. although some growth took place at concentrations up to 0·1 mg./ml. From our experiments the leucocyte test would appear

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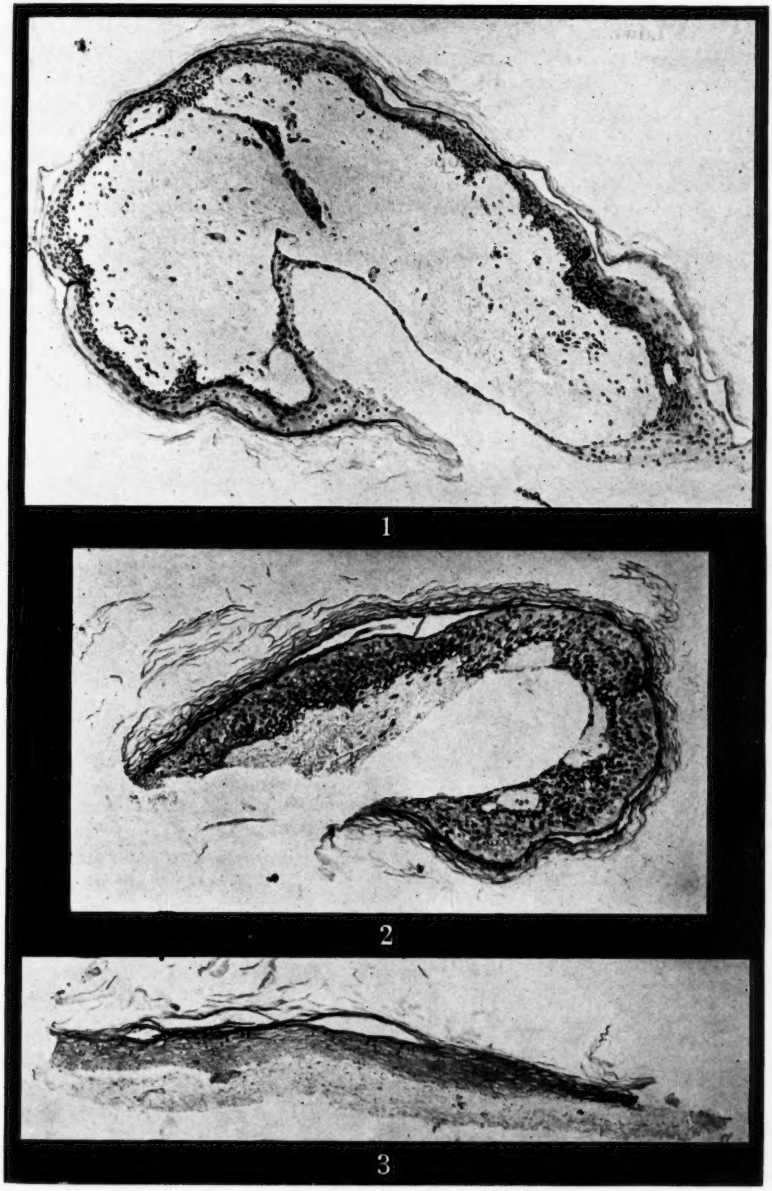
DESCRIPTION OF PLATES.

FIG. 1.—Normal culture. Epidermis with normal appearance, and a continuous layer of migrating epidermal cells over the cut surface of the dermis.

FIG. 2.—Inhibited culture. Almost complete failure of migration. Numerous vacuoles are present in the epidermis.

FIG. 3.—Necrotic culture. Absence of migration, and degeneration of epidermal cells. There is partial separation of epidermis from dermis.



Cruickshank and Lowbury.

to be less sensitive than tissue culture, as no abnormal effect was noted at concentrations of 0·08 mg./ml. and toxicity was apparent only at 0·24 mg./ml.

The main interest in the findings is their relation to the amounts of pyocyanin found on burns colonised with *Ps. pyocyanea*. Our experiments showed that in burns producing green discoloration of dressings the concentration of pyocyanin in the exudate was between 0·008 mg./g. and 0·31 mg./g. In each instance the concentration was above that which might be expected to produce toxic effects upon the skin *in vitro*. It seems reasonable to suppose that dressings soaked with the green pigment may act as a reservoir of pyocyanin which hinders the repair of burned skin and the success of skin grafts. Moreover the reservoir is continuously replenished by the growth of *Ps. pyocyanea*, so that absorption into the circulation and dilution by fresh exudate will not necessarily diminish the concentration of pyocyanin on the burned surface.

It must be emphasised that the rôle of the local toxicity of pyocyanin in the pathogenicity of *Ps. pyocyanea* is postulated only with regard to surface infections. The animal experiments suggested that in deeper infections the local production of pyocyanin was not of significance. This conclusion is based on the absence of any response to the intradermal injection of pyocyanin and the fact that there was no difference in the size of the lesions caused by pyocyanin-producing and by non-pyocyanin-producing strains.

SUMMARY.

Experiments were made to determine the toxicity of pyocyanin to tissue cultures of human skin and to human leucocytes. Pyocyanin was found to be toxic to skin *in vitro* at levels above 0·0024 mg./ml. and to leucocytes at levels above 0·08 mg./ml.

Estimates were made of the concentration of pyocyanin present on 5 burns infected with *Ps. pyocyanea*. The concentrations ranged from 0·008 to 0·31 mg./g.

The concentrations found on human burns were all capable of causing toxic effects on skin *in vitro*. The leucocytes were less sensitive, being affected only by the highest concentrations found in burn exudates.

Intradermal injection of pyocyanin into guinea-pigs caused no local lesion. The lesions produced by the intradermal injection of pyocyanin producing and non-pyocyanin producing strains of *Ps. pyocyanea* did not differ in size.

These findings are discussed in relation to the observed ill-effects produced by *Ps. pyocyanea* on human burns.

We wish to thank Mr. J. R. Cooper, F.I.M.L.T., and Mr. H. Street for technical assistance. We are also grateful to Mr. D. M. Jackson, F.R.C.S., and the nursing staff of the Medical Research Council Burns Unit, Birmingham Accident Hospital for their co-operation.

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THE GROWTH AND RESPIRATION OF LEPTOSPIRA IN SOLID OR SEMI-SOLID MEDIA WITH SPECIAL REFERENCE TO DINGER'S PHENOMENON.

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MOST work on the cultivation of leptospira described in the literature is related to growth in fluid or semi-solid media. Inada, Ido, Hoki, Kaneko and Ito (1916) at the outset used media which had proved successful with spirochaetes in Noguchi's hands, and they covered them with fluid paraffin. Ito and Matsuzaki (1916) who describe the pure cultivation of *Spirochaeta icterohaemorrhagiae*. (*Inada*) used both semi-solid media consisting of equal parts nutrient agar and blood of an infected guinea-pig, or 1 part gelatin with 2–4 parts guinea-pig blood, and fluid media consisting of 1 part human or ox serum with 1 part water. The gelatin medium was the most successful but they point out that covering with fluid paraffin is unnecessary. It was, however, Noguchi's extensive investigation (1918) that set the line which subsequent work on cultivation has mostly followed. He recommended rabbit serum 10–25 per cent, diluted with Ringer's solution and preferably containing about 0·3 per cent of agar. He also states that the leptospira are strictly aerobic and he observed a haze of growth at the surface, sharply demarcated below, in media containing agar. Further he found that even 1·5 per cent peptone caused marked inhibition of growth. He also noted a remarkable tolerance of the leptospira to the substitution for Ringer of various diluents of the serum, *e.g.*, filter-sterilised sewage water.

In our own observations on the growth of leptospira in semi-solid or solid media, two well defined manifestations of growth other than the development of a diffuse haze of turbidity have been noted. The first, which was obtained at the outset of our studies, was the appearance after 2 weeks' cultivation of loose fluffy colonies remote from the surface of the medium, which are illustrated in Fig. 1 and 2 and closely resemble those of *Spirochaeta microdentium* seen in a culture of that micro-organism prepared by Noguchi and sent to one of us by the kindness of the late Simon Flexner in 1913. These appearances occurred in a variety of less favourable media such as routine autoclaved nutrient agar diluted 1/6 or 1/9 with water prior to the addition of 10 per cent of human serum, but rarely in the medium adopted later in which the second visible form of growth was obtained with great regularity.

This medium is described below, and a culture on it is represented in Fig. 3. The appearance in this plate is that of double disc formation, which is usually developed about the 10th day of incubation at 30°. The upper disc commonly appears on the 3rd–7th day, is situated 3–10 mm. below the surface and is as sharply demarcated below at first as the second and lower one in Fig. 3. If the tube is cut across at the base and the cylinder of solid medium is allowed to slip

out, it can be shown in preparations made with material cautiously sucked up with a Pasteur pipette that there are scanty or moderately numerous leptospira in the medium above the disc, but below it none or so few that their presence is most probably attributable to unavoidable flaws in the method of making such preparations, whereas great numbers are found within the zone of the disc. Fig. 4 presents something of both disc and colony formation.

So far as we are aware, although we cannot claim to have made a minute survey of the very extensive literature on leptospira, the first of these phenomena of growth has not been described, unless by Fletcher (1928), who published a photograph of a somewhat similar appearance which he obtained when growing leptospira in semi-solid media in the presence of antiserum to which they were susceptible. It is clear, however, that something like the second has been observed from time to time. A ring of denser growth slightly below the surface in semi-solid media is described in Noguchi's early paper (1918). Fletcher (1928) published a photograph showing a ring of denser growth sharply demarcated below at a much lower level than we usually obtain and with considerable turbidity throughout the upper half of the tube above it. Bessemans and Thiry (1930) also describe the development of a dense ring of growth near the bottom of the tube in cultures in which the serum had settled at the bottom of the tube and never been properly mixed. Dinger (1932) made a very full description and study of the phenomenon exactly as we have seen it, in a paper published in Dutch and illustrated by good photographs.

The phenomenon seems for the most part to have escaped attention, and although reference to Dinger's work is made in van Thiel's (1948) monograph it is neither mentioned nor illustrated in any textbook which we have seen. A photograph of it has, however, been published in a recent Spanish monograph (Busquets, 1951) without reference to the earlier work. It has also been the subject of further study by Lawrence (1951). Dinger's attention was drawn to this phenomenon when investigating the remarkable persistence of virulence which some of his leptospira had shown after 15 months' subculture *in vitro*. This he finally attributed to the superiority of a medium containing agar; he was mainly comparing a modified Noguchi medium (5–6 per cent nutrient agar in 10 per cent rabbit's serum dissolved in tap water) with a modified Vervoort (1923) medium (0·1 per cent peptone in buffer with 10 per cent rabbit's serum), and he was inclined to relate the superiority of the former to the phenomenon of disc formation.

Dinger found that growth diminished or failed at O_2 tensions below 40 mm. and above 300 mm., and that the depth of the disc from the surface could be varied in a culture according to the pressure of O_2 within that range to which it was exposed at the surface. These observations obviously suggested that the leptospira develop the discs of massed growth at levels of optimum oxygen tension and that to this extent they are not strictly aerobic. He did not, however, accept this conclusion, but found the explanation of the development of these narrow zones of massed growth in the diffusion of metabolic products, which would otherwise inhibit growth, into the underlying medium, the disc forming at the farthermost point to which an adequate O_2 supply had penetrated. His main ground for this conclusion was that there was no increased growth in fluid media exposed to low but still adequate O_2 tensions.

Lawrence's observations (1951) mainly confirm Dinger's and he inclined to

accept his conclusion with regard to the explanation of the phenomenon. In support he points out that the growth of leptospira in Vervoort's medium is increased if the culture is grown in a cellophane bag immersed in excess of that medium. In electrometric observations which coincided satisfactorily with O/R indicator findings, Lawrence determined potentials in the zone of growth of approximately + 0·06, while in the lower parts of the tube devoid of growth levels of — 0·05 to — 0·1 were found. He was unable to relate the level of the band of heavy growth to CO_2 concentrations as Wilson (1931) had done in cultures of *Brucella abortus* which develop somewhat similar bands of condensed growth at levels some way beneath the surface.

EXPERIMENTAL

Our own observations, arising from the investigation of a strain (*Leptospira leeds*) obtained from the urine in a case of " abacterial " cystitis (Czekalowski and Horne, 1951 ; Czekalowski and McLeod, 1953), were carried out mainly in media consisting of nutrient broth which had not been autoclaved, but filtered through a Maassen or Chamberland candle for sterilisation after dilution with 7–9 parts of water and incorporation of 10 per cent of a suitable rabbit serum. This filtrate was heated at 56° for 30–60 min. and molten 4 per cent agar was then added so as to bring the final concentration of agar to 0·8–1 per cent. The medium was inoculated with 0·2–0·25 ml. of culture, mixed, and after solidification in the cold, incubated at 30° in 6 ml. amounts in 6″ × $\frac{5}{8}$″ (15·2 × 1·6 cm.) test tubes. It will be noted in the first place that these observations were made in solid rather than " sloppy " agar. Disc formation was readily obtained with higher concentrations of agar although at 2 per cent or above it was not well developed. In these conditions our observations coincided very nearly with those of Dinger, including double or multiple ring or disc formation at lower levels in the tubes when incubation was continued for periods longer than 7 days (Fig. 3).

Many different peptones were tried in the nutrient broth and at one time or another the phenomenon was obtained with all. As, however, the influence of peptone will be discussed in detail in a subsequent paper on the nutrition of leptospira, we will not consider it here. Dinger states that he was unable to obtain the disc phenomenon with a few of his strains. This has not been our experience as we have obtained it with each of 12 strains tested, which have included *L. icterohaemorrhagiae, L. canicola, L. biflexa* and antigenically degraded strains like " McVady ". It is probable that Dinger was dealing with strains recently isolated from disease in man, although this is not stated. With the exception of *L. leeds* all our strains had been in subculture for considerable periods and even *L. leeds* had been subcultured for at least 4 months before we made observations on the disc phenomenon. There appeared to us, however, to be a relatively sharp distinction amongst leptospira by this test. The group of strains which agglutinated with a serum prepared from rabbits against *L. leeds*— the homologous strain, the strain " McVady ", and two strains " Jackson " and " McIntyre " which had been obtained from a collection maintained at the Edinburgh Royal Infirmary but which no longer agglutinated with anti-*L. icterohaemorrhagiae* serum—all produced coarser discs 3–5 mm. from the surface, whereas " Wijnberg " and other strains agglutinating with anti-serum for *L.*

icterohaemorrhagiae developed finer discs at 6–10 mm. beneath the surface. This difference might be interpreted in the sense that the latter had a lower O_2 toleration, but it is more probably explained by the slower development of the *L. icterohaemorrhagiae* strains and deeper penetration of an adequate O_2 concentration by the time that growth was well developed.

Observations Bearing on the Respiration of Leptospira.

Oxidation-reduction potentials.

Although our observations on this subject are less extensive than those of Lawrence, they agree with his conclusions. Thus when haemoglobin was incorporated in the agar it was oxidised to a brownish colour (? methaemoglobin) above the disc and reduced below. Methylene blue behaved similarly, remaining coloured in and above the disc and colourless below it. Where two discs were formed the reduction was limited to the part of the medium below the second disc. Janus Green was partially reduced (green to pink) above the disc, and completely reduced to pink below it, but never developed the colourless phase. Pheno-safranine was not reduced. As the reductions described only indicate slightly negative O/R potentials, such as will be developed by the medium itself provided that O_2 is excluded, and since material aspirated from the zone beneath the disc after cutting across the base of the tube is devoid of leptospira, it would seem that these forms are obligate aerobes and that they lack a mechanism for active reduction such as is put into operation by the anaerobes and to a lesser degree by most micro-aerophilic and facultatively anaerobic forms.

Catalase, hydrogen peroxide and oxidase.

Evidence of catalase activity was sought by covering cultures which had developed frank discs with weak solutions of H_2O_2 and allowing them to stand for 30–60 min. There was no development of gas although this should have been very readily detected by splitting of the agar in the vicinity of the dense growth in the disc area had catalase been produced. Controls with known catalase-positive bacteria grown in similar conditions on the same medium showed active gas formation.

To elicit evidence of peroxide formation well grown tubes were covered with a layer of Schönbein's reagent, but no trace of a blue colour was obtained. It has been observed by McLeod, Gordon and Pyrah (1923) that haematin agar (chocolate) is a peculiarly delicate indicator for H_2O_2, developing a green colour in its presence, but we were unable to explore this test as no active growth of leptospira in media containing heated blood was obtained.

Bacterial oxidase is believed to correspond to cytochrome oxidase, and the most delicate test for it is to run a dilute solution of tetramethyl-*p*-phenylene diamine-hydrochloride over surface colonies and off again, a positive reaction being indicated by the development of a deep purple colour in the colonies as they dry. It is stated categorically, however, by Kolle and Hetsch (1938) that surface colonies of leptospira on solid media have never been obtained and we have not found mention of it in the more recent literature. In our own work in which the medium described for obtaining disc formation was used as slopes with approximately a 1 per cent agar content, and copious inoculation was made to

the condensation water, we observed with a coarsely growing leptospira an upward spread of a delicate film of growth as in Fildes' method (1925) for the isolation of *Cl. tetani*, but nothing like colony formation.

It was not therefore possible to carry out this test under ideal conditions, and in any case it was found that the medium used promoted rather rapid oxidation of the diamine at a surface exposed to air. If, however, the reagent was added in amount just sufficient to cover the surface of a deep tube of medium in which the disc phenomenon had developed about 10 mm. below the surface, a narrow line of purple sometimes appeared at the disc level as well as at the surface, the intervening area remaining relatively pale. Controls of uninoculated medium showed purple only at and immediately below the surface.

This may indicate the presence of oxidase activity, but in view of the possibility of various artefacts, more elaborate work would be needed for confirmation.

Utilisation of Dinger's Phenomenon in Work on the Cultivation of Leptospira.

In order to determine how far this phenomenon was unique, cultures of a wide range of bacteria in media and conditions similar to those described above and also in the same medium tinted with haemoglobin from laked blood were made. Tubes containing cultures of leptospira and uninoculated medium served as controls.

The appearances in similar solid culture media of other bacteria are set out in Table I for contrast with those of leptospira which have already been described and illustrated. It is clear that none of these bacteria produces appearances at all closely resembling those obtained with the leptospira and hence such appearances can be accepted as a valuable indicator of good growth in cultures of leptospira; much more so than the development of turbidity in fluid media. A second practical point is that so long as well defined discs persist in such cultures it is possible to obtain subcultures when growth has failed with material taken from fluid cultures of similar age.

We have not made a sufficiently extensive experiment on this point with rigid controls to justify more than the suggestion that these observations taken in conjunction with Dinger's warrant an extended trial of this type of culture in the preservation of leptospira.

DESCRIPTION OF PLATES.

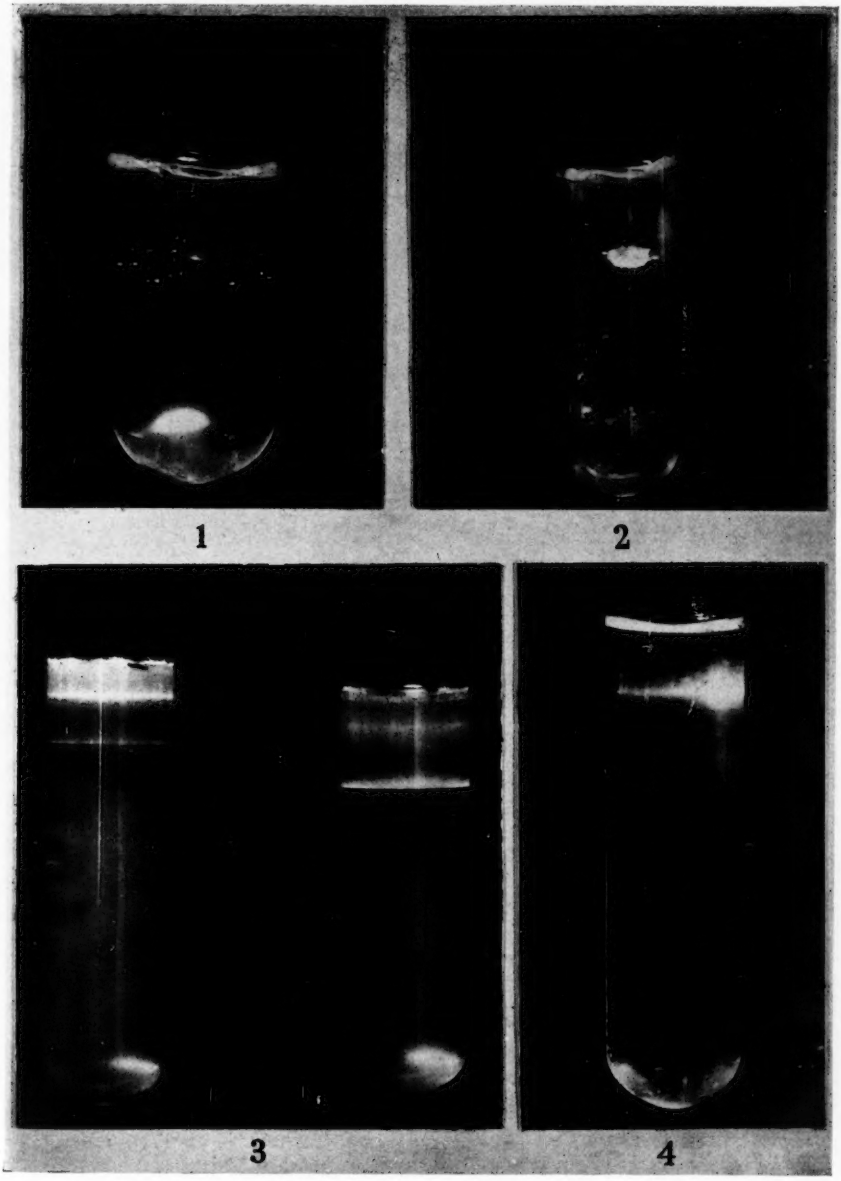
FIG. 1.—Small or medium colonies appearing in a wide zone between the upper $\frac{1}{3}$ and lower $\frac{2}{3}$ of the medium. These appeared in the third week of cultivation at 30°.

FIG. 2.—One large opaque colony of irregular aspect about 12 mm. below surface and a few smaller loose fluffy ones near base of tube.

FIG. 3.—Shows usual appearance after 10 days' culture of a coarsely growing leptospira which has been heavily inoculated to the medium described in the text. Both cultures show two zones of growth; the second to appear is the lower one, and it gives the characteristic disc being very sharply demarcated below. On the 4th or 5th day the upper one would be solitary and show a similar appearance.

FIG. 4.—Culture showing a blend of disc formation and development of colonies.

The surface opacity appearing in some tubes and the large lenticular opacities seen near the base of some tubes are artefacts due to technical difficulties in this kind of photography.



Czekalowski, McLeod and Rodican.

TABLE I.—*Respects in which Appearance of Cultures of other Bacteria differ from those of Leptospira in*

Micro-organism.	10 per cent serum in dilute broth and 0·8 per cent agar.	The same tinted with haemoglobin.
Control, no inoculation	Slight diffuse turbidity, *i.e.*, no change	Oxidised to a light brown throughout
Bact. coli *Salm. paratyphi B* *Salm. typhi*	Heavy turbidity at surface and for 9 mm. below it; slight uniform turbidity beyond this	Uniform reduction except for 3 mm. at surface
Staphylococcus aureus	As last but zone of heavy turbidity only extends 3 mm. from surface	As last
Streptococcus (*a*) *pyogenes* (*b*) *faecalis* (*c*) *salivanius*	All show two or more ill-defined zones of increased turbidity in the first 12 mm. below surface: (*c*) also shows granular defined colonies throughout	(*a*) As control (*b*) Lowest 3 or 4 mm. reduced (*c*) Lower half reduced
C. diphtheriae (*a*) *gravis* (*b*) *intermedius* (*c*) *mitis*	All show turbidity due to fine discrete colonies for 3 mm. below surface and then heavier turbidity for 3 mm. further. (*c*) also showed many fine discrete colonies throughout depth	Partial reduction only in all
C. hoffmanni	Heavy turbidity for first 3 mm. at surface	As last
Neisseria meningitidis	Poorly developed growth	Poor growth
Pseudomonas pyocyanea	Marked surface turbidity for 2–3 mm.	Marked turbidity for 2 mm. at surface. Half of tube below this reddish, ? oxidised: rest reduced
Proteus vulgaris	Surface to 3 mm. marked turbidity; lessening turbidity in upper ⅓ of tube	Surface–3 mm. heavy turbidity; below lesser turbidity and reduction
Vibrio cholerae	Surface–2 mm. turbidity marked; below this 6 mm. clear; remainder of depth shows granular turbidity greater than control	Surface–6 mm. marked turbidity; remainder moderate opacity and slight reduction
Vibrio metchnikovi	Surface zone of turbidity deeper than with last and depth of tube clear	As last but suggestion of double zone of turbidity near surface
Haemophilus influenzae	Slight zone of turbidity about 4 mm. below the surface; poor growth	As control
Haemophilus para-influenzae	Surface–depth of 4 mm. marked turbidity; many discrete colonies throughout	Surface–4 mm. very turbid; rest reduced
Shigella shigae	Many discrete granular colonies throughout	Not investigated
Brucella abortus		
Strain no longer CO_2-sensitive	Heavy zone of turbidity 2–5 mm. below surface but not sharply defined beneath	" "
Strain CO_2-sensitive	Poor growth; ill defined zone of turbidity 5–8 mm. from surface	" "

DISCUSSION.

Our observations fall completely into line with those of Noguchi, of Dinger and of Lawrence, since they indicate leptospira as strict aerobes in the sense that they will not grow in the absence of free O_2. It seems to us that, this notwithstanding, the phenomena observed indicate a preference for an O_2 tension

TABLE II.—*Inhibitory Effect of KCN Solution on Growth of* Leptospira canicola (" *Aldgate* ")

KCN dilution.	Development of discs and depth at which they appeared.	
	4th day.	10th day.
1–1000	*nil*	*nil*
1–3000	,,	,,
1–10000	,,	Hazy disc at 8–11 mm.
1–30000	Well defined disc	Well defined discs at 7 and at 11–12 mm.
1–100,000	,, ,, ,,	Well defined discs at 7, 9 and 10 mm.
Control, no cyanide	,, ,, ,,	Well defined discs at 7 and 9 mm., faint disc at 11 mm.

Series was duplicated using *Bact. coli* as inoculum; no evidence of inhibition of growth was observed in any tube.

Medium was 10 per cent rabbit serum in meat extract diluted 1/8 containing 0·1 per cent peptone and 0·8 per cent agar.

pH of all media after addition of cyanide was within the range 7·5–8·0.

less than the atmospheric and that in this respect these organisms differ from recognised aerobes such as many species of the genus *Bacillus*, the vibrios and the meningococcus. If they prefer an atmospheric or a greater O_2 tension they should on Dinger's argument grow best at the surface. Further his diagram of their growth in media exposed to high O_2 tensions shows the disc of maximum growth so near the bottom of the tube that there is little space for diffusion of the hypothetical inhibitory growth products beneath the disc. Lastly, secondary rings or discs are often so near the first as to be in the zone which might be expected to contain a maximum of such products.

They appear rather to be micro-aerophilic forms with a special respiratory mechanism distinct from most other such species which reduce actively and produce traces of H_2O_2, and from most aerobes which are equipped with catalase and oxidase. They are, nevertheless, peculiarly cyanide-sensitive as is indicated in Table II, in which they are compared in this respect with *Bacterium coli*. It seems likely, therefore, that some cytochrome mechanism plays a part in their respiration.

CONCLUSIONS.

That leptospira form discs of dense growth a few mm. below the surface of solid and semi-solid media has been confirmed. An account of this phenomenon, which assists the recognition of viable cultures, might with advantage be included in text-book descriptions.

The respiratory mechanism of leptospira is peculiar in that although they require gaseous O_2, and to this extent are strict aerobes, they probably in a limited sense are micro-aerophilic. They are cyanide-sensitive, and resemble a small group of bacteria, of which the best known is the dysentery bacillus of *Shiga*, in lacking catalase, having slight reducing activity, and in failing to produce recognisable traces of H_2O_2.

We are much indebted to Mr. G. Eaves for help in translating Dinger's paper from the Dutch, to Dr. R. Holman for help with some of the experiments, to Mr. T. Varley for much painstaking work in the preparation of media and to the Medical Research Council for a grant in aid of expenses.

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EXCRETION OF URINARY PEPSINOGEN (UROPEPSIN) IN CASES OF PEPTIC ULCERATION AND OF MACROCYTIC ANAEMIA.

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ALTHOUGH much work has been done in the United States and in Germany (Gottlieb, 1924*a*, *b* ; Bucher, 1947 ; Merten, 1948 ; Mirsky, Block, Osher and Broh-Kahn, 1948 ; Podore, Broh-Kahn and Mirsky, 1948 ; and others) there only seem to be two references (Hirschowitz, 1953 ; Eastcott, Fawcett and Rob, 1953) in the British medical literature to the excretion of urinary pepsinogen. In view of the claims made for this enzyme, some further comments may be of interest.

Urinary pepsinogen, which appears to be identical with gastric pepsinogen, is the proteolytic enzyme of urine most active at a pH of approximately 2. It is not derived from reabsorption from the gastric lumen but diffuses direct from the peptic cells of the gastric mucosa into the blood stream. The concept of an endocrine origin receives additional support from the work of Spiro, Reifenstein and Gray (1950), confirmed by Eastcott *et al.* (1953), who showed that the injection of ACTH in experimental subjects led to a marked increase in the 24 hr. excretion of urinary pepsinogen. They also showed that compound E can increase the excretion of the enzyme in subjects suffering from adrenal insufficiency.

Opinions regarding the value of urinary pepsinogen assays in diagnosis differ widely : Mirsky *et al.* (1948) state that peptic ulcer subjects excrete pepsinogen at twice the rate of healthy people. Janowitz, Levy and Hollander (1950) found the excretion in duodenal ulcer cases to be four times the normal, whereas gastric ulcer cases showed normal values. Eastcott and his co-workers noted a persistent increase in the excretion of the enzyme in cases of duodenal ulceration and they agree with Janowitz and his colleagues that the assay has a diagnostic value. Hirschowitz (1953), however, considers the estimation to be of no diagnostic value in this condition. Bucher (1947) found that high protein diets increased the output of pepsinogen while Merten (1948) found a diminished excretion in malnutrition, chronic phthisis and in famine oedema.

Some workers (Strauss, 1923 ; Gottlieb, 1924*a* ; Teschendorf, 1927 ; Farnsworth, Speer and Alt, 1946 ; Mirsky *et al.*, 1948 ; Sylvest, 1949) have noted the absence of pepsinogen from the urine of patients suffering from pernicious anaemia, while Janowitz and Hollander (1950) found a normal excretion in two cases of megaloblastic anaemia due to non-tropical sprue.

The writer investigated the excretion of urinary pepsinogen in 76 cases suffering either from peptic ulceration or from macrocytic anaemia.

Method of Assay.

A modified form of Anson's (1938) method was used to measure pepsinogen in urine. Twenty-four hr. specimens of urine were collected and 1 ml. of acidified urine was incubated at 37° for 1 hr. with 5 ml. acidified 2 per cent haemoglobin substrate. Enzyme action was

terminated by the addition of 10 ml. 4 per cent trichloracetic acid. A blank was also set up. The filtrates from the test and blank were treated with Folin and Ciocalteu's reagent (1927) and then made alkaline with Na_2CO_3. The colours were compared in a " Gallenkamp " photoelectric colorimeter. A tyrosine solution (150 μg./ml.) was used as a standard. Owing to the lack of uniformity among units of pepsinogen, the results were expressed in mg. tyrosine produced by the 24 hr. volume of urine.

Excretion in Peptic Ulceration.

The excretion of urinary pepsinogen was estimated in 65 cases, comprising 26 normal subjects, 25 with a duodenal and 14 with a gastric ulcer. The results are shown in the Figure.

Excretion in Cases of Macrocytic Anaemia.

The excretion of pepsinogen was examined in 11 cases. There were 6 cases of pernicious anaemia and 3 of macrocytic anaemia without achlorhydria, 1 case of macrocytic anaemia with histamine-fast achlorhydria but which, on haematological grounds, was thought to be a nutritional megaloblastic anaemia and not

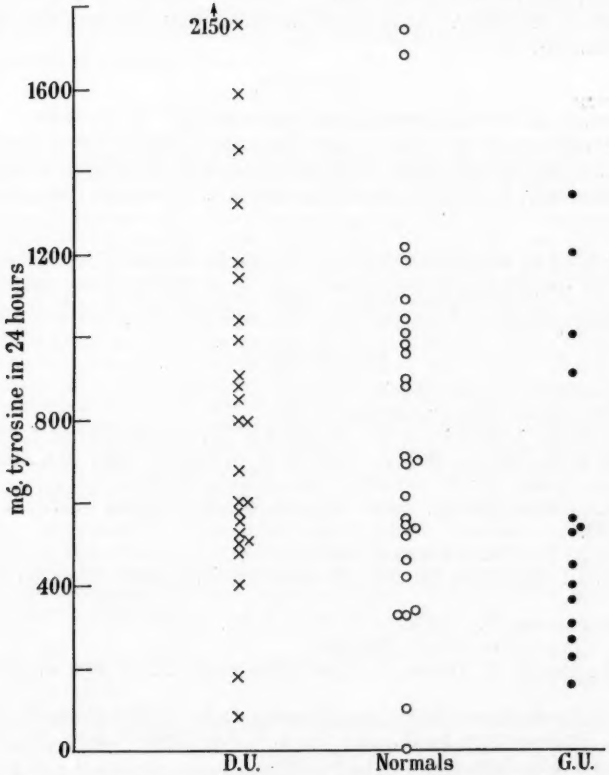

FIGURE.—Urinary pepsinogen excretion in cases of duodenal ulcer, normal subjects, and cases of gastric ulcer.

the true Addisonian type, and 1 case of aplastic anaemia where the bone marrow showed megaloblastic degeneration.

The results were as follows :

(1) There was no excretion of urinary pepsinogen in the cases suffering from pernicious anaemia.

(2) The other cases of macrocytic anaemia showed a normal excretion.

DISCUSSION.

An examination of the Figure shows a great degree of overlap between the three groups, and the writer is in agreement with Hirschowitz (1953) that the assay has no diagnostic value in cases of peptic ulceration. The normal variation in the amount of urinary pepsinogen excreted is so great that the writer believes that only the presence or absence of the enzyme has any diagnostic significance. Urinary pepsinogen assays could be of value in the differentiation of pernicious anaemia from other macrocytic anaemias, and the estimation would be particularly useful in cases where gastric analyses were impossible. Urinary pepsinogen is comparatively stable, even in alkaline urines, and a delay of 48 hr. would not affect the assay. It is clear, however, that the test would be of very limited application.

SUMMARY.

The excretion of urinary pepsinogen was examined in 76 cases.

The assay appears of no value in the diagnosis of peptic ulcerations.

The enzyme was absent from the urine of cases of pernicious anaemia.

The assay would be a valuable diagnostic aid in certain cases of macrocytic anaemia.

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THE MOVEMENT OF LYMPHOCYTES.

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AMOEBOID motion of lymphocytes *in vitro* was described by Ranvier (1875), Jolly (1898*a*, *b*, *c*; 1903), Wolff (1901), Hirschfeld (1901), Neumann (1903) and Askanazy (1905). More recent observations have been made by McCutcheon (1924), Lewis and Webster (1921) and Lewis (1931, 1933). Dixon and McCutcheon (1935), Schade and Mayr (1930*a*,*b*) and Coman (1940) found that lymphocytes did not exhibit chemotaxis *in vitro* under conditions in which chemotaxis could readily be demonstrated in granulocytes. In the present experiments the photomicrographic trace method previously described for demonstrating chemotaxis in granulocytes and monocytes (Harris, 1953*a*, *b*) was modified for the study of the movement of lymphocytes.

MATERIALS AND METHODS.

The lymphocytes were obtained from the thoracic duct of the rabbit. The animal was starved for 24 hr. before operation to reduce the fat content of the lymph as much as possible. The duct was cannulated in the abdomen just above its exit from the cisterna chyli. In most of the animals the duct at this site was a large single trunk, but, occasionally, there was a leash of smaller vessels leading to the main trunk; in such cases one of the larger tributaries of the duct was cannulated. About 3 ml. of lymph were collected into 30 ml. of sterile normal saline. The dilution of the lymph in this way prevented its clotting. The dilute lymph was centrifuged at 220 g for 3 min. to separate the cells from any fat still present in the suspension. The supernatant was discarded and the cells were re-suspended in 3 ml. of Hanks' solution (Hanks, 1948) containing 20 per cent of aged rabbit serum. To 9 drops of this cell suspension was added 1 drop of rabbit plasma, which had been collected in chilled vessels without anticoagulant and stored frozen until required. A fragment of the material to be tested was deposited on the surface of a clean slide, and a film of the plasma-containing cell suspension was laid down between the slide and a coverslip, as previously described (Harris, 1953*a*). In this way a plasma coagulum about 20μ deep was obtained, which incorporated both the test object and a random dispersion of lymphocytes. The preparation was sealed with paraffin wax and incubated at 37°.

The movement of the lymphocytes relative to the test object was recorded photographically by continuous exposure under dark ground illumination. The lymphocytes were less brilliant than granulocytes or monocytes, so that the intensity of the illumination had to be slightly increased. In other respects the optical system was the same, and Kodak "Micro-file" Panchromatic film was again suitable. Each negative was exposed continuously for about half an hour.

RESULTS.

The nature of the cells.

The cells obtained from the rabbit thoracic duct were typical "large" or "small" lymphocytes. The large lymphocytes varied greatly in size and some of them had the staining characteristics usually attributed to immature cells.

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Numerous mitotic figures were seen among the large lymphocytes, but none was seen among the small lymphocytes. It was often difficult to be certain, on morphological grounds alone, that some of the large lymphocytes were not monocytes, but differences in the behaviour of these two cells *in vitro*, which are discussed later, showed clearly that these large cells were not monocytes. A fixed and stained preparation of the cells obtained from the thoracic duct is shown (Fig. 1).

All the cells were alive when they were incorporated in the plasma film. This was shown by the fact that all of them, when suspended in a medium containing the supravital dye, neutral red iodide, were able to concentrate the dye in their cytoplasm. The dye was segregated in no more than 6–8 vacuoles; in this respect the cells behaved in the manner shown to be characteristic for lymphocytes by Simpson (1922).

Movement of the lymphocytes.

Although all the cells were alive, only 20–30 per cent were motile. Some were amoeboid from the beginning and others, which were rounded and stationary when incorporated in the plasma film, became amoeboid on incubation. The majority, however, remained rounded and stationary throughout the whole observation period of 5 or 6 hr. The proportion of motile cells usually began to diminish after the first $1\frac{1}{2}$ hr. of incubation, and at the end of 6 hr. only an occasional motile cell was seen. By this time some of the stationary cells showed well-defined nuclear membranes, and were presumably dead. Many of the cells showed amoeboid forms without actual locomotion. Both large and small lymphocytes were capable of movement, although the small lymphocytes were more active. There was great variation in the rate of movement of different cells, and of any one cell from time to time. The character of the amoeboid movement also varied. Some of the cells, both large and small, moved with the nucleus in advance and a single projection of cytoplasm extending behind, as described by Clark, Clark and Rex (1936). Sometimes a constriction ring could be distinguished in the cytoplasm, and progress appeared to be effected by passage of the nucleus through this ring, as described by Lewis (1931). Other cells were greatly elongated and moved in the direction of their long axis without any obvious constriction ring; others were irregular in shape, putting out pseudopodia in different directions as they moved. The paths taken by the cells were quite haphazard, although it was not uncommon for a small lymphocyte to move some distance in an almost straight line.

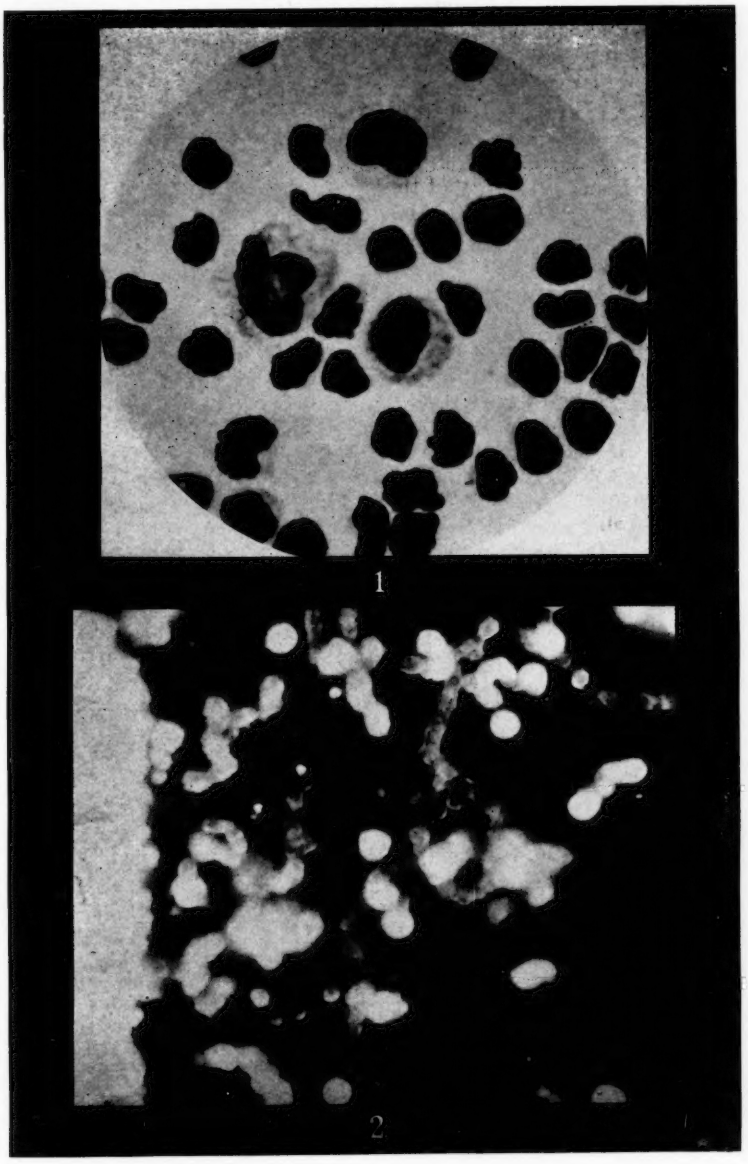
The photographic records produced by moving lymphocytes showed tortuous irregular traces, which varied in width and density. An occasional fairly straight trace was made by one of the small lymphocytes. Scattered among the traces were numerous round dots which were produced by the stationary cells (Fig. 2).

The tenuous attachment of lymphocytes to the matrix in which they move

DESCRIPTION OF PLATES.

FIG. 1.—Sample of large and small lymphocytes obtained by cannulation of thoracic duct. (May-Grünwald-Giemsa. × 760.)

FIG. 2.—Pattern traced out by lymphocytes in the presence of a clump of *Myco. tuberculosis* (H37Ra). The clump is on the left. Traces show no tendency to converge on the test object. The round dots represent stationary cells. Exposure 30 min. (× 500.)



Harris.

has been pointed out by Lewis (1931). In the present experiments this characteristic of lymphocytes was most noticeable. The cells did not adhere at all to the glass surfaces between which they moved, and they made only slight contact with the fibrin mesh of the plasma film. The inability of lymphocytes to adhere to glass enabled a clear distinction to be made between the large lymphocytes and monocytes. Living monocytes from the rabbit peritoneal cavity, incubated at 37° in a slide-cell chamber, soon became closely adherent to the floor of the chamber. While adherent in this way, they assumed amoeboid forms, but were not actively motile (Harris, 1953*b*). If the chamber was inverted the cells were not dislodged. Under similar conditions lymphocytes from the rabbit thoracic duct did not adhere to the floor, and some of them became actively motile almost immediately. If the chamber was inverted the cells at once fell to the bottom.

The effect of micro-organisms.

Clumps of the following bacteria were tested: *Myco. tuberculosis* (H37Ra), *Ery. monocytogenes*, *C. diphtheriae*, *Salm. typhi*, *H. pertussis*, *Staph. albus*, *Staph. aureus*, *Str. pyogenes*, *B. anthracis* and *Kl. pneumoniae*. Starch grains were also tested. In no case could any sign of chemotaxis be seen; the lymphocytes continued to move in a completely random way as long as they remained motile, and there was no accumulation of cells in or around the test object. The photographic trace record produced in the presence of a clump of *Myco. tuberculosis* (H37Ra) is shown (Fig. 2); the lymphocytes show no tendency to converge on the test object. In the presence of the same organism monocytes produced records in which all the traces within a certain range of the test object converged directly on it (Harris, 1953*b*, Fig. 3). As previously described, the monocytes rapidly ingested the clump of bacteria, but phagocytosis by either large or small lymphocytes was never observed.

The effect of cells parasitized with virus.

Clumps of immature mouse testis cells, infected with the G.D. VII strain of mouse encephalomyelitis virus at a concentration of 1 LD_{50} per cell, were not chemotactic for lymphocytes.

The effect of autolysed and partially digested tissues.

Fragments of rabbit muscle and liver which had been allowed to autolyse under aseptic conditions for 3–24 hr., and others which had been partially digested with pepsin or trypsin, were tested. These tissue fragments were not chemotactic for lymphocytes, nor did the lymphocytes adhere to the fragments when they came into contact with them in the course of random migration.

DISCUSSION.

None of the substances which had been shown to be chemotactic *in vitro* for granulocytes and monocytes, was found to be chemotactic for lymphocytes under similar conditions; nor were cells parasitized with virus or clumps of *H. pertussis*, which were tested because of their apparent association with lymphocytes in pathological lesions. The results of the present investigation are thus in agreement with those of all other workers who have studied chemotaxis in lymphocytes.

It is possible that the right conditions for demonstrating chemotaxis in lymphocytes have not yet been found, or the right test objects; but the consistently negative findings of all authors suggest the possibility that the lymphocyte does not respond to chemotactic stimuli. Dixon and McCutcheon (1935) suggested that the differences in this respect between lymphocytes and the other leucocytes might be due to differences in the cell surfaces. The present investigations confirm that such differences do exist. In varying degree, both granulocytes and monocytes adhere to glass at 37°, and, during locomotion, adhere to the matrix in which they move. Lymphocytes, on the other hand, cannot be made to adhere to glass, and they make only slight contact with the matrix in which they move. If the capacity of a cell to exhibit chemotaxis does depend on its surface properties, this difference between the surface properties of the lymphocyte and those of the other leucocytes might help to explain the apparent absence of chemotaxis in the lymphocyte.

SUMMARY.

The photomicrographic trace method previously used for demonstrating chemotaxis in granulocytes and monocytes was adapted for the study of lymphocytes obtained from the rabbit thoracic duct.

The lymphocyte did not react chemotactically towards any of the substances tested. These comprised a variety of micro-organisms, starch grains, cells parasitized with virus, autolysed tissues, and tissues partially digested by pepsin or trypsin.

Certain differences were noted between the surface properties of the lymphocyte and those of the other leucocytes.

It is a pleasure to thank Sir Howard W. Florey for his help in this work. Thanks are also due to Mr. A. A. Argyle for technical assistance and to Mr. B. H. Glass for the photography. The mouse testis cells parasitized with encephalomyelitis virus were provided by Dr. F. K. Sanders.

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THE EFFECT OF *KLEBSIELLA AEROGENES* AND *KLEBSIELLA CLOACAE* POLYSACCHARIDES ON HAEMAGGLUTINATION BY AND MULTIPLICATION OF THE INFLUENZA GROUP OF VIRUSES.

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SUBSTANCES which inhibit viral haemagglutination have been shown to be of two types, those which act primarily on the virus particle itself and those which affect the red blood cells. Thus the mucopolysaccharide found in normal serum and first described by Francis (1947) appears to inhibit viral haemagglutination by combining with the virus particles and preventing their union with red cells. A large number of similar inhibitors have been described (Burnet, 1951) and in general following incubation with active virus they progressively lose their power to inhibit haemagglutination. This loss of activity is thought to be due to the action of a viral mucopolysaccharidase.

Another group of substances inhibiting viral haemagglutination consists mainly of plant and bacterial polysaccharides. Members of this group have been found to retain their activity in the presence of virus and in some cases these substances have been thought to be active by combination with the red cell. Thus Keogh, North and Warburton (1947) have shown that red cells treated with a polysaccharide-containing fraction from *Haemophilus influenzae* resisted agglutination by influenza virus. Kozinski (1951) obtained similar results with polysaccharide-containing autolysates from several strains of *Salmonella typhi*. Ginsberg, Goebel and Horsfall (1948*b*) showed that the polysaccharide derived from *Klebsiella pneumoniae* type B also appeared to act by a combination with the red cell. They further showed that although red cells rendered resistant to haemagglutination by treatment with inhibitory polysaccharide failed to absorb mumps virus they would absorb and elute influenza A and B viruses like normal cells. No explanation was given of these apparently contradictory findings. Polysaccharides from *Kl. pneumoniae* types A, B and C were found to inhibit the multiplication of the mumps virus *in ovo* (Ginsberg, Goebel and Horsfall, 1948*a*), but types A and C had no effect on haemagglutination. These authors suggested that inhibition of viral multiplication and haemagglutination by polysaccharides are not closely related phenomena and that the effect on multiplication may be due to the polysaccharide inhibiting cellular metabolic systems necessary for viral proliferation. No explanation was offered for the inhibition of haemagglutination by these polysaccharides.

In the following experiments *Klebsiella aerogenes* and *Klebsiella cloacae* polysaccharides have been shown to combine with red cells and to inhibit their agglutination by viruses. The surface charge on these cells is shown to be increased and a theory to explain the inhibitory action of the polysaccharides is based on this fact.

MATERIALS AND METHODS.

Viruses.

PR8. Influenza A.
Barclay. Influenza A prime.
Lee. Influenza B.
NDV. Newcastle disease virus, Herts strain.
Mumps virus, Enders strain.

All these strains were used in the form of allantoic fluids harvested from chick embryos inoculated by the method described by Beveridge and Burnet (1946).

Saline.

The saline used throughout was 0·85 per cent sodium chloride in distilled water buffered at pH 7·0 with 0·05 M phosphate.

Fowl red cells.

Blood was withdrawn from the wing vein into citrated saline. The cells were washed 3 times with saline, stored at 4° and were discarded if not used in the next 4 days.

Klebsiella *strains.*

Those prefixed A were isolated in this department by Dr. J. P. Duguid, and those prefixed N were obtained from the National Collection of Type Cultures. Full details of the properties of *Kl. aerogenes* strains A1, A3, A3 " Sl ", and A3 " O " and *Kl. cloacae* strains N5920 and N5936 are given in Wilkinson, Duguid and Edmunds (in preparation). *Kl. aerogenes* strains A4 and A29 are capsulate, slime-forming mucoid bacteria identical in their biochemical reactions to A3. Dr. P. N. Edmunds of this Department has shown that in the system of Edwards and Fife (1952), strains A1, A3 and A3 " Sl " are type K54. Strains A4, A29, N5920 and N5936 are separate types not corresponding to any of those of Edwards and Fife, but they have been typed by Edmunds (personal communication).

Immune sera.—Washed suspensions of living bacteria were used to immunise rabbits. Serum was obtained 7 days after the last of a series of intravenous injections, and the titre estimated by capsule-swelling and agglutination tests.

Preparation of bacterial polysaccharides.

Each strain was grown on a medium containing excess sugar to give a maximum production of polysaccharide (Duguid and Wilkinson, 1953). A saline suspension of cells was then treated as follows :

Preparation of slime polysaccharides.—Cells were centrifuged down and the polysaccharide precipitated from the supernatant by the addition of 1 per cent sodium acetate and 3 volumes of acetone. The precipitate was dissolved in water and de-proteinised by Sevag's chloroform butyl alcohol method (Sevag, 1934). The solution was then dialysed for 24 hr. against running tap water, followed by treatment with anionic and cationic exchange resins. The polysaccharide was then re-precipitated, washed with acetone and ether, and dried.

Preparation of capsule and slime polysaccharides.—The cell suspension was adjusted to pH 7·0, boiled for 30 min. and centrifuged. The polysaccharide was precipitated from the supernatant by the method described above.

Virus haemagglutination titrations.

Dilutions of infected allantoic fluid were prepared in buffered saline. The amount of virus which in a volume of 0·25 ml. produced partial haemagglutination 1 hr. after the addition of 0·25 ml. of 0·5 per cent suspension of fowl red cells was taken to equal one haemagglutinating dose.

Titration of haemagglutination-inhibiting activity of polysaccharide.

Volumes containing 0·25 ml. of a 0·75 per cent suspension of red cells were added to 7 × 1 cm. tubes and left at room temperature for 30 min. Only those tubes in which the red cells showed no traces of agglutination or lysis were used. To these tubes was then added 0·25 ml. of the required polysaccharide dilution and the tubes were shaken before being left

for a further 30 min. at room temperature. In this way the effect of each polysaccharide on the red cells was determined. On no occasion did the polysaccharides agglutinate the red cells. The virus dilution then added was such that 0·25 ml. was calculated to contain 4 haemagglutinating doses. The tubes were then well shaken and left for 1 hr. The titration end point was taken as the last tube showing no haemagglutination.

Virus infectivity titrations.

Serial 10-fold dilutions of infected allantoic fluid were made in phosphate buffered saline (pH 7·0), and 0·1 ml. of the desired dilution of virus was inoculated intra-allantoically through a small drill hole. The allantoic fluid was tested for its power to cause haemagglutination. End-points (E.I.50) were calculated according to the method of Reed and Muench (1938).

Electrophoretic mobilities and rates of sedimentation.

The electrophoresis apparatus was adapted from that described by Anderson and Mackie (1939). It consisted of four U-tubes (A, B, C and D) graduated in mm. on each side arm and having an internal diameter of 1·1 cm. They were connected in series by agar bridges of length 13 cm. and internal diameter 0·4 cm. The bridges contained 1·36 per cent KH_2PO_4, 1·42 per cent Na_2HPO_4, 1·26 per cent NaCl and 2 per cent agar. Further agar bridges connected the tubes to the anode vessel containing 5 per cent $CuSO_4$ and to the cathode vessel containing 1 per cent NaCl. The red cells were made up in 15 per cent suspensions in 0·1 M phosphate buffer pH 7·0 made isotonic by the addition of 0·26 per cent NaCl, and 10 ml. of this suspension were placed in each tube. Buffer was carefully pipetted up to a standard mark while the other limb was temporarily closed. This procedure was reversed and buffer was added to a standard mark in the other limb giving a total volume of 15 ml. Agar bridges were dipped into the buffer in the tubes to a depth of 0·5 cm. The platinum electrodes were connected up to a mains supply of 230 volts D.C. giving a current of 13·5 m. amps passing through the apparatus. The experiment was carried out at room temperature and measurements were made of the level of red cells in both limbs of each tube at 10 min. intervals for 90 min. The difference in the rate of displacement of the boundaries in the two U-tubes limbs was taken as a measure of the electrophoretic mobility. The average of the readings was calculated and the result expressed as μ/min. The average rate of sedimentation under gravity in the two limbs was also measured and calculated as μ/min.

RESULTS.

The Effect of Treatment with NaOH on the Haemagglutination-inhibitory Properties of Kl. aerogenes *and* Kl. cloacae *Polysaccharides.*

When the polysaccharides were prepared by the method described above none were found to inhibit haemagglutination by the 5 viruses tested. However, after treatment with 0·25 N NaOH for 3 hr. at 37° followed by neutralisation with HCl, several of the polysaccharides showed marked powers of haemagglutination inhibition while others remained inactive. The results are summarised in Table I.

Each titration was carried out at least 3 times using red cells from different fowls on each occasion. The results were remarkably constant and the infrequent discrepancies small.

Maximal inhibitory action was shown by each preparation following 3 hr. treatment at 37° or 1 hr. at 56° with 0·25 N NaOH. Further treatment at 37° or 56° for as long as 24 hr. did not alter the inhibitory titre.

Treatment of A3 polysaccharide with 0·25 N HCl for periods of 1–24 hr. at 37° followed by neutralisation did not give an active product.

In the remainder of the paper, those polysaccharides treated with 0·25 N NaOH for 3 hr. at 37° have an OH added in the brackets describing the type of polysaccharide, *i.e.*, *Kl. aerogenes* A3 capsular and slime polysaccharide without soda treatment is represented A3 (CS) and after soda treatment as A3 (CS,OH).

TABLE I.—*The Effect of* Klebsiella *Polysaccharides after NaOH Treatment on Haemagglutination by the Influenza Group of Viruses.*

Source of polysaccharide. C = capsule. S = slime. I = intracellular.	Concentration of polysaccharide (μg./ml.) which just inhibited haemagglutination by the following viruses:				
	PR8.	Barclay.	Lee.	Mumps.	NDV.
Kl. aerogenes A1 (CS)	333	166	166	166	333
" " A3 (CS)	2·5	2·5	2·5	2·5	2·5
" " A3 (C)	2·5	2·5	2·5	2·5	2·5
" " A3 (S)	2·5	2·5	2·5	2·5	2·5
" " A4 (CS)	5	2·5	1·2	5	5
" " A29 (CS)	1·5	6	3	6	6
" " A3 "Sl" (S)	N*	N	N	N	N
" " A3 "O" (I)	"	"	"	"	"
Kl. cloacae N5920 (S)	"	"	"	"	"
" " N5936 (S)	"	"	"	"	"

* N = non-inhibitory.

The Interaction of Inhibitory Polysaccharide with Virus and Red Cell.

Experiments were designed to show whether the inhibitory action of the polysaccharide was directed primarily on the red cell or on the virus particle and also if prolonged contact with virus lowered the inhibitory titre of the polysaccharide.

The effect of incubating A3 (CS,OH) polysaccharide with PR8.

The effect on the polysaccharide.—Fresh allantoic fluids from 13-day-old chick embryos inoculated 48 hr. previously with PR8 virus were pooled and gave a haemagglutination titre greater than 1000. To 3 tubes 1·5 ml. of infected allantoic fluid and 0·5 ml. of a sterile 1 mg./ml. solution of A3 (CS,OH) were added. One tube was immediately heated to 100° for 15 min., this treatment having been found adequate to inactivate the virus without altering the inhibitory titre of the polysaccharide. The other 2 were incubated at 37° and 18° respectively for 16 hr. before being similarly inactivated. The inhibitory titres of all 3 preparations were found to be the same, indicating that the polysaccharide was unaltered by the virus.

The effect on the virus.—Immediately before the contents of the tubes described above were inactivated by heating, 0·25 ml. aliquots were removed and the haemagglutination titre of each determined. The end-points did not differ significantly from the controls. The controls consisted of pooled infected allantoic fluid to which was added 0·5 ml. saline in the place of A3 (CS,OH) solution, and which were similarly incubated. In the titration of the polysaccharide—virus mixtures, haemagglutination was shown only in the higher dilutions and at a point greater than the inhibitory concentration of the polysaccharide. These results indicate that no irreversible combination or interaction takes place between the virus and the polysaccharide since the virus can be freed from the inhibitory action of the polysaccharide by diluting a mixture of the two.

The effect of inhibitory polysaccharide on the red cell.—If these polysaccharides act on the red cell and combine with it, as has been shown by Ginsberg *et al.*, (1948*b*) to be the case with *Kl. pneumoniae* polysaccharide, then they should show

resistance to haemagglutination after washing the cells free from uncombined polysaccharide.

To 3 ml. of a 1 mg./ml. solution of A3 (CS,OH) was added 3 ml. of a 10 per cent suspension of red cells. The suspension was left for 3 hr. at room temperature and frequently shaken. The cells were then centrifuged and washed 4 times with saline. The packed cells were made up into a 0·5 per cent suspension and 0·25 ml. of this suspension was added to doubling dilutions of PR8 virus of known haemagglutinating titre in 0·25 ml. volume. The polysaccharide-treated cells resisted 600 haemagglutinating doses of virus. This result suggests that the inhibitory action of the polysaccharide is primarily due to some irreversible change on the red cell surface.

The following experiment was carried out to determine whether there is a loss of polysaccharide as determined chemically after treatment of a solution of polysaccharide with red cells.

Six doubling dilutions of A3 (CS,OH) polysaccharide were prepared in saline. The initial dilution contained 500 μg./ml. and the final dilution contained 15 μg./ml. To each of a series of tubes containing 5 ml. of the polysaccharide dilutions and also to a control tube containing 5 ml. saline was added 5 ml. of a 10 per cent suspension of red cells. The mixture was shaken frequently while being held at room temperature for 3 hr. and then left overnight at 4°. The red cells were packed by centrifugation and the supernatant removed. The cells were then washed twice with 2 ml. aliquots of saline by re-suspension and centrifugation and the washings added to the original supernatant. The volume was then made up to 15 ml. with saline and the polysaccharide content estimated by the anthrone method (Seifter, Dayton, Novic and Muntwyler, 1950).

The results are shown in Table II. It can be seen that 1 ml. of a 5 per cent suspension of red cells will absorb from 5–11 μg. A3 (CS,OH) from solution, the

TABLE II.—*The Absorption of Polysaccharide (A3(CS,OH)) by a 5 per cent Suspension of Red Cells.*

Initial concentration of polysaccharide μg./ml.	250	125	62·5	31·3	15·6	7·8
Final concentration of polysaccharide μg./ml.	240	114	57	24	7·5	2·5
Amount of polysaccharide absorbed by red cells μg./ml.	10	11	5·5	7·3	8·1	5·3

amount being largely independent of the initial concentration of polysaccharide. These results suggest that there is a certain fixed amount of polysaccharide which will combine irreversibly with the red cell. It must be emphasised, however, that the method of estimation has its limitations, particularly at the highest and lowest concentrations.

If the polysaccharide combines with the red cell and forms a layer on the cell surface, antiserum which reacts and forms a precipitate with it should agglutinate red cells so sensitized.

Suspensions of red cells (10 per cent) were made up in saline solutions containing 1–2 mg./ml. of a series of polysaccharides and left overnight at 4°. The cells were then centrifuged and washed 4 times with saline. The serum was inactivated at 60° for 30 min. and doubling dilutions were prepared in 0·2 ml. of saline. To each tube was added 0·2 ml. of 0·2 per cent treated red cell suspension and the tubes were well shaken before being incubated overnight at 4°. The results are shown in Table III, the end point being expressed as the reciprocal of the highest

TABLE III.—*The Agglutination of Polysaccharide-treated Red Cells with Antiserum.*

Sensitising polysaccharide.	Highest serum dilution giving partial agglutination of polysaccharide-treated red cells (reciprocal).			
	Pooled normal rabbit.	Anti-A3.	Anti-A4.	Anti-N5936.
None	4	4	8	4
A3 (CS)	8	4	—	—
A3 (CS,OH)	4	512	4	—
A4 (CS)	4	8	8	—
A4 (CS,OH)	4	8	512	—
A1 (CS)	4	8	—	—
A1 (CS,OH)	8	512	—	—
A3 " Sl " (S)	4	16	—	—
A3 " Sl " (S,OH)	4	1024	16	—
N5936 (S)	4	—	—	16
N5936 (S,OH)	4	—	—	16

dilution showing agglutination. (The antiserum prepared against A3 suspensions precipitates with A1, A3 and A3 " Sl " polysaccharides. It was found that all polysaccharides used, both before and after NaOH treatment precipitated with the homologous antiserum produced against washed suspensions of each organism).

From Table III it will be seen that marked agglutination of red cells with dilute antiserum will only take place when the cells have been sensitised with the homologous NaOH-treated polysaccharide. This suggests that only following NaOH treatment are the polysaccharides able to combine with the red cell and also that the inhibition of haemagglutination is correlated with the absorption of the polysaccharide on the red cell surface. However, since A3 " Sl " (S,OH) combines with the red cell but does not alter its agglutinability by virus, it seems that absorption of a polysaccharide on the red cell surface is not enough in itself to cause inhibition of haemagglutination, and that some other factor is also responsible.

The Absorption of Virus by Polysaccharide-treated Red Cells, and its Elution from these Cells.

Polysaccharides may inhibit because they combine with the virus receptors on the red cell surface, thus preventing the virus acting as a link between red cells and so inhibiting haemagglutination. If this is so, an inhibitory polysaccharide such as A3 (CS,OH) should prevent absorption whereas a non-inhibitory polysaccharide such as A3 " Sl " (S,OH) should allow absorption having left the virus receptors free, and combined with some other part of the red cell surface. The following experiments were carried out to test this hypothesis.

Direct measurement of absorption and elution of virus.

Suspensions of red cells were made up to 10 per cent in saline and in a solution of A3 (CS,OH) in saline (2 mg./ml.). They were left overnight at 4° when the cells were centrifuged and washed 3 times with saline. The red cells were then made up in 10 per cent suspension in undiluted PR8 virus-infected allantoic fluid having a haemagglutination titre of 1200. The suspensions were frequently shaken while being held at room temperature. At intervals 0·5 ml. was with-

drawn with chilled pipettes from each tube and centrifuged at 1–2°. The supernatants were then titred by haemagglutination for their virus content and the results shown in Fig. 1 reflected the ability of normal and polysaccharide-treated red cells to absorb and elute PR8 virus. A similar experiment was carried out using undiluted mumps virus-infected allantoic fluid having a haemagglutination titre of 200, the results also being shown in Fig. 1.

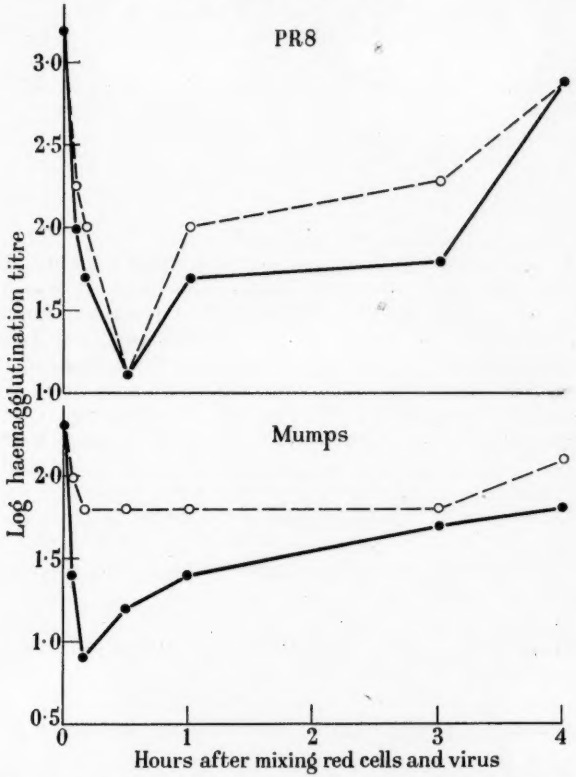


FIG. 1.—The absorption of virus by normal and polysaccharide-treated red cells, and its elution from these cells.

Top graph, PR8 virus; bottom graph, mumps virus. —●—●—●— normal red cells; —○—○—○—A3(CS,OH) polysaccharide-treated red cells.

From Fig. 1 it can be seen that PR8 virus is absorbed by and eluted from polysaccharide-treated red cells in almost exactly the same way as it is from normal cells. Since the polysaccharide-treated cells are highly resistant to haemagglutination by PR8 virus it seems that the mechanism of haemagglutination inhibition by this polysaccharide is not due to its presence on the red cell preventing primary red cell-virus union. However, it does seem from Fig. 1 that polysaccharide-treated red cells are much less able to absorb mumps virus than normal

red cells. This effect would explain why such cells are resistant to agglutination by the mumps virus.

Electron microscopy of normal and polysaccharide-treated laked red cells before and after virus treatment.

Since polysaccharide-treated red cells resistant to haemagglutination appear to absorb virus like normal red cells, it was of interest to determine whether the presence of virus could be demonstrated by electron microscopy on polysaccharide-treated laked cells.

Using a tube test it was found that a concentrated suspension of PR8 having a haemagglutination titre with normal red cells of 2000 failed to agglutinate a 1 per cent suspension of saponin-laked red cell ghosts (Dawson and Elford, 1949) in dilutions greater than 1/300. When the laked cells were soaked in A3 (CS,OH) and then washed, agglutination was inhibited even with a 1/2 dilution of the virus suspension. These treated cells were agglutinated by a 1/256 dilution of anti-A3 serum showing that the A3 polysaccharide had combined with the laked cells. It is of interest to note that although normal laked red cells sometimes gave auto-agglutinable suspensions in saline, they were stabilised and settled out normally following treatment with A3 (CS,OH).

Laked cells were made up into a 10 per cent suspension in a saline solution of A3 (CS,OH) (2 mg./ml.) and left overnight at 4°. After washing 4 times with saline the laked cells were then added to a 1/10 dilution of PR8 in saline to give a 5 per cent suspension. The mixture was maintained at 0° for 45 min. when an equal volume of chilled 0·1 per cent osmic acid in saline was added. The fixed cells were washed 3 times with distilled water, mounted on the grid membrane, dried and shadowed at an angle of 15° with Au/Pd. They were then examined in the electron microscope. Controls included normal laked cells with and without added virus.

From Fig. 2, 3 and 4 it can be seen that normal and polysaccharide-treated laked cells both absorb PR8 virus to approximately the same degree.

A similar experiment with mumps virus showed that polysaccharide-treated laked cells had a greatly reduced power to absorb mumps virus compared with normal control cells.

The Effect of Kl. aerogenes *and* Kl. cloacae *Polysaccharides on the Surface Charge of Red Cells.*

In the light of these results, any explanation of the mechanism of polysaccharide inhibition of haemagglutination by PR8 virus must allow for the fact that the resistant cells are still capable of absorbing this virus.

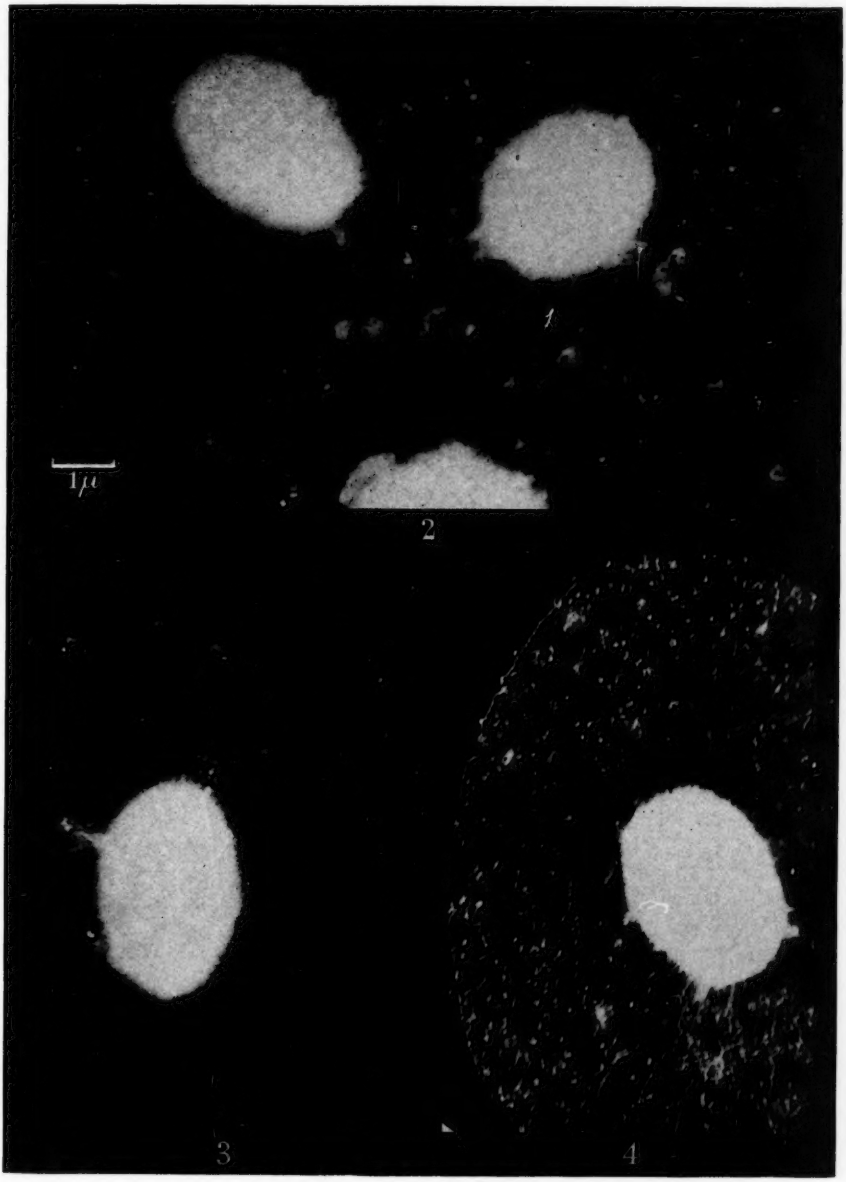
The possibility that the polysaccharide acted by increasing the surface charge of the red cell was investigated. If the surface charge was increased it is conceivable that the resultant increase in the mutual repulsion of treated red cells

DESCRIPTION OF PLATE.

FIG. 2.—Electron micrograph of normal laked fowl red cells treated with PR8 virus showing virus particles on the cell membrane. (× 9,000.)

FIG. 3.—Normal laked fowl red cell. (× 9000.)

FIG. 4.—Laked fowl red cell treated with inhibitory A3 (CS,OH) polysaccharide and with PR8 virus showing virus particles on the cell membrane. (× 9000.)



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would be sufficient to reduce or even eliminate the possibility of red cell–virus–red cell links being formed, the latter being assumed to be necessary for haemagglutination.

The acid agglutination point.

The acid agglutination point of a red cell varies with its surface charge. By suspending polysaccharide-treated cells in buffered saline at various pH values, it was possible to determine whether the acid agglutination point had decreased or increased and therefore if the cells' surface charge had been raised or lowered. Red cell suspensions (10 per cent) were prepared in saline solutions of different polysaccharides (1–2 mg./ml.) and left overnight at 4°. The cells were then centrifuged, washed 4 times with saline and made up into 10 per cent suspensions in saline. To 0·025 ml. of each red cell suspension were added 0·5 ml. volumes of a range of buffers varying by 0·2 pH units. The buffers were 1/10 dilutions in saline of McIlvaine's buffer (0·1 M citric acid and 0·2 M disodium hydrogen phosphate—pH range 2·2–8·0).

The results shown in Table IV indicate that only those polysaccharides which inhibit haemagglutination lower the acid agglutination point to a marked extent.

TABLE IV.—*The Acid-Agglutination Points of Normal and Polysaccharide-Treated Red Cells in McIlvaine's Buffers.*

Sensitising polysaccharide (no NaOH treatment).	pH at which agglutination just occurs.	Sensitising polysaccharide (NaOH treated).	pH at which agglutination just occurs.
None	4·6	—	—
A3 (CS)	4·6	A3 (CS,OH)*	3·8
A4 (CS)	4·4	A4 (CS,OH)*	3·4
A3 " Sl " (S)	4·4	A3 " Sl " (S,OH)†	4·4
N5936 (S)	4·6	N5936 (S,OH)‡	4·6
A1 (CS)	4·6	A1 (CS,OH)*	4·0
A29 (CS)	4·6	A29 (CS,OH)*	3·8

* Inhibitory. † Absorbed but non-inhibitory. ‡ Not absorbed.

This suggests that the presence of an inhibitory polysaccharide on the red cell surface increases the negative charge and thus lowers the acid agglutination point and therefore presumably the isoelectric point of the cell.

The electrophoretic mobilities and rates of sedimentation of normal and polysaccharide-treated red cells.

If the surface charge of a red cell is altered by polysaccharide, it may be possible to demonstrate changes in the electrophoretic mobility of these cells.

Red cells were made up into 20 per cent suspensions in 2 mg./ml. solutions of various polysaccharides and left overnight at 4°. The cells were then washed 3 times with saline and once with the buffer used for suspension in the electrophoresis apparatus. Polysaccharide-treated cells were placed in tubes A and C and normal cells in tubes B and D. The experiment was then repeated with the same cells after they had been washed once and re-suspended in 10 ml. fresh buffer, the U-tubes being interchanged. The average value of the 4 tubes was

taken and electrophoretic mobility of the polysaccharide-treated cells compared with the control.

The results shown in Table V indicate that only those polysaccharides which inhibit haemagglutination cause an increase in the electrophoretic mobility (EPM)

TABLE V.—*The Electrophoretic Mobilities and Rates of Sedimentation of Normal and Polysaccharide-Treated Red Cells.*

Polysaccharide used.	U-tube.	Electrophoretic mobility (μ/min.). Polysaccharide treated cells (PC).	Average.	Normal cells (NC).	Average.	Rate of sedimentation (μ/min.). Polysaccharide treated cells (PC).	Average.	Normal cells (NC).	Average.
A4 (CS,OH)	A	5·0	5·5	3·9	3·9	13·0	12·4	18·3	17·1
	B	6·5		3·6		12·5		18·0	
	C	5·0		4·8		12·8		15·4	
	D	5·5		3·5		11·3		16·6	
		Difference PC from NC + 41 per cent.							
A3 (CS,OH)	A	5·5	6·0	3·0	3·2	6·1	6·1	18·8	17·0
	B	6·0		2·6		6·4		18·4	
	C	6·9		3·9		6·3		15·0	
	D	5·5		3·2		5·5		16·0	
		Difference PC from NC + 87 per cent.							
A3 "Sl" (S,OH)	A	3·5	3·2	2·7	3·4	19·0	17·7	16·5	15·2
	B	2·4		3·1		16·5		17·2	
	C	3·2		3·7		16·4		15·7	
	D	3·8		4·1		19·1		11·3	
		Difference PC from NC − 6 per cent.							
A1 (CS,OH)	B	3·3	4·4	3·6	3·7	18·1	15·0	19·0	17·0
	C	5·3		3·3		14·5		14·5	
	D	4·5		4·1		12·5		17·5	
		Difference PC from NC + 19 per cent.							
N5936 (S,OH)	A	4·1	4·2	4·2	3·9	17·3	18·1	18·7	18·8
	B	4·4		3·6		18·9		19·0	
		Difference PC from NC + 8 per cent.							

and therefore in the surface charge. It should also be noted that the rate of sedimentation of inhibitory polysaccharide-treated red cells is retarded approximately in proportion to the degree of increase in the EPM.

The importance of the surface charge in viral haemagglutination can be seen from this and the previous section. Thus none of the polysaccharides which have previously been shown to be unabsorbed, *i.e.*, those with no NaOH treatment and also N5936 (S,OH), produced any appreciable change in the acid agglutination point (AP) or in the EPM. On the other hand those polysaccharides capable of absorption and causing inhibition of agglutination all lowered the AP and increased the EPM. It can also be seen that A1 (CS,OH) which is not so effective in inhibiting haemagglutination lowers the AP to a smaller extent than A3 (CS,OH) and A4 (CS,OH). The importance of the lowering of the AP and raising of EPM, both the result of an increased surface charge, can be seen in the case of A3 "Sl" (S,OH). This polysaccharide, although it is absorbed by the red cell, causes no lowering of the AP or increase in the EPM and is non-inhibitory.

The Effect of Kl. aerogenes *and* Kl. cloacae *Polysaccharides on the Multiplication* in ovo *of the Influenza Group of Viruses.*

Ginsberg *et al.* (1948, *a*, *b*) found that the polysaccharide of *Kl. pneumoniae* type B inhibited both haemagglutination and multiplication by the mumps virus. Since the polysaccharides studied in this investigation are similar in many respects to those used by these authors, it was of interest to determine their effect on virus multiplication.

An inoculum of 0·25 ml. polysaccharide solution was introduced into the allantoic sac of at least six 11-day-old chick embryos (8-day-old embryos in the case of mumps virus). After leaving the embryos for 1½ hr. at 37° a second inoculum containing 100 E.I.50 doses of virus in 0·1 ml. saline was introduced intra-allantoically. As a control, a second batch of at least 6 embryos was inoculated initially with 0·25 ml. of saline and after 1½ hr. at 37° with 100 E.I.50 doses of virus. Embryos inoculated with PR8 virus and NDV were incubated at 36° for 48 hr. and those inoculated with mumps virus for 5 days at 35°. Following overnight chilling at 4° the allantoic fluid of each embryo was tested for its content of haemagglutinins, this being taken as a criterion of virus growth.

As will be seen from Table VI, none of the polysaccharides brought about a significant inhibition of viral multiplication.

TABLE VI.—*The Effect of* Klebsiella *Polysaccharides on the Multiplication of PR8, Mumps Virus* in ovo.

Virus.	Polysaccharide preparation.	Amount of polysaccharide per embryo (mg.).	No. of embryos alive after incubation was complete.	Average haemagglutination titre of each group (reciprocal).
PR8	None	—	5/6	1092
„	A3 (CS)	1	6/12	910
„	A3 (CS,OH)	1	5/6	1080
„	A4 (CS)	1	4/6	800
„	A4 (CS,OH)	1	7/10	960
„	A1 (CS,OH)	0·5	5/6	1280
„	A29 (CS)	1	6/6	1173
„	A29 (CS,OH)	1	4/6	1280
„	N5920 (S)	1	6/6	1065
NDV	None	—	4/12	1200
„	A3 (CS)	1	5/8	1150
„	A3 (CS,OH)	1	6/8	983
Mumps	None	—	6/6	83
„	A3 (CS)	1	18/20	90
„	A3 (CS,OH)	1	15/20	98

DISCUSSION.

It is now generally believed that haemagglutination by the influenza group of viruses is the result of virus particles acting as links between red cells and in this way aggregating the cells into lattices. Good evidence for the validity of this theory has been brought forward by Anderson (1947) to explain haemagglutination by NDV. If this mechanism is accepted, the fact that red cells treated with inhibitory polysaccharide and rendered resistant to agglutination by PR8 may still absorb this virus to an undiminished extent, suggests that the stage of the

process inhibited must be the linking of virus-coated red cells. The only feasible means by which this could be brought about is by a mechanism which prevents the red cells coming close enough together to allow virus absorbed on one cell to link with vacant receptors on another. In this study good correlation has been shown to exist between the ability of a polysaccharide to inhibit haemagglutination and its power to increase the surface charge of the red cell.

The action of NaOH in activating potential inhibitory polysaccharides to combine with the red cell is not known. It is possible that the mild treatment used brings about an uncoiling of the molecule and reveals polar groups previously masked. This hypothesis is supported by the findings of Kuhn, Künzle and Katchalsky (1948) who showed that the addition of alkali brought about the uncoiling of a macromolecule (polymethylacrylic acid) by the repulsion of the ionised groups. The effect alkali may have on polysaccharides is very variable and not well understood, viscosity changes, depolymerisation, and removal of acetyl groups having all been shown to occur as a result of its action.

It now seems clear that inhibition of viral haemagglutination by polysaccharides of the type investigated in this study cannot be used as a means of screening substances for their therapeutic usefulness in the treatment of the corresponding viral infection. Ginsberg *et al.* (1948 *a*, *b*) found that *Kl. pneumoniae* type B polysaccharide inhibited both haemagglutination and multiplication *in ovo* by the mumps virus but came to the conclusion that these phenomena were not due to a single mechanism. Whether the increased surface charge on the red cell presents an impenetrable electrostatic barrier to the mumps virus or whether inhibition is due to the polysaccharide masking or combining with the mumps receptor on the red cell has not yet been determined.

SUMMARY.

Polysaccharides from strains of *Kl. aerogenes* and *Kl. cloacae* have been shown to inhibit haemagglutination by the influenza—mumps—NDV group of viruses. These polysaccharides only show this effect after mild treatment with NaOH followed by neutralisation.

The inhibitory effect is due to firm combination of NaOH-treated polysaccharide with the red cell.

Red cells resistant to haemagglutination by PR8 virus absorbed and eluted this virus much like normal red cells, but those rendered resistant to mumps virus did not absorb the virus to any appreciable extent.

Red cells rendered resistant to viral haemagglutination by polysaccharide had a lower acid agglutination point, a lower rate of sedimentation, and an increased electrophoretic mobility.

These results reflect an increased surface charge on the red cells and provide an explanation of the mechanism of inhibition of viral haemagglutination by bacterial polysaccharides.

None of the polysaccharides tested inhibited the multiplication of this group of viruses *in ovo*.

We are greatly indebted to Professor T. J. Mackie for much helpful advice and criticism. Our thanks are also due to Dr. P. N. Edmunds for the supply of type-specific immune sera.

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A METHOD FOR INFLUENZA ANTIBODY ABSORPTION.

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THE introduction of the haemagglutination inhibition test offered a very convenient means of studying the antigenic characters of influenza viruses. However, very few attempts have been made to establish qualitative and quantitative antigenic relationships among these viruses by antibody absorption techniques as such studies are severely hampered by difficulties in the methods at present available. Important contributions have been made by Friedewald (1944) and Hirst (1952) using absorbed sera. In both instances the sera were absorbed with large amounts of concentrated virus. Fazekas de St. Groth (1949) suggested that specific antibody could be absorbed from influenza antiserum with periodate-treated fowl erythrocytes to which influenza virus has been adsorbed (PVR-cells). By a modification of Fazekas de St. Groth's technique, Burnet (1952) recently showed that when inhibitor-sensitive influenza virus (indicator virus) is adsorbed to fowl erythrocytes and these cells are subsequently treated with the receptor destroying enzyme (RDE) of culture filtrate of *Vibrio cholerae*, only about one-third of the virus is removed. According to Burnet the virus remaining on the cells is irreversibly bound and is not removed either by RDE, mucoid inhibitors or immune serum.

Burnet's observation seemed to offer a method for antibody absorption which might be more convenient than the techniques of Friedewald (1944) and Hirst (1952), and the present paper reports an attempt to devise such a technique along with tests of its validity.

MATERIALS AND METHODS.

Virus suspensions.—Four strains were selected for the present study: three A strains, PR8 (Francis, 1934), A/England/1/51 (Isaacs and Andrewes, 1951) and A/Missouri/302/53 (received from Dr. M. R. Hilleman) and one B strain, Crawley (Dudgeon, Stuart-Harris, Glover, Andrewes and Bradley, 1946). The virus suspensions were obtained by inoculating 0·05 ml. of infected allantoic fluid diluted 10^{-3} into the allantoic cavity of 11-day-old chick embryos. The eggs were incubated at 35° for 48 hr., chilled at 4° for 1 hr. and the allantoic fluids harvested. The fluids were dialysed for 48 hr. against a large volume of 0·001 M borate buffer (Clark, 1920) at 4° and then centrifuged at 2500 r.p.m. for 5 min. and stored at − 70° until used.

The Crawley strain was made inhibitor-sensitive (indicator virus) by adjusting to pH 8·0 with phosphate buffer and heating at 56° for 30 min. The viruses PR8, A/England/1/51 and A/Missouri/302/53 were made inhibitor-sensitive by addition of Calgon (sodium hexametaphosphate) to a final concentration of 0·0002 M, adjusting to pH 8·0 and heating at 56° for 30 min. (Edney, 1949; Burnet and Edney, 1952).

The virus suspensions were made isotonic by addition of 0·15 ml. of 1·0 M NaCl per ml. dialysed suspension.

Immune sera.—Immune ferret sera were treated with RDE to destroy non-specific inhibitors of haemagglutination by a modification (Isaacs and Bozzo, 1951) of the method of

van der Veen and Mulder (1950). The sera were diluted 1 in 5 in acetate buffer, pH 6·2, and 500 units of RDE were added per ml. undiluted serum. The sera were maintained overnight in a waterbath at 37° and then heated at 62° for 30 min. to inactivate RDE. Heating at this temperature had no detectable effect on the antibody titre.

Receptor destroying enzyme(RDE).—Partially purified RDE was prepared from culture filtrates of strain 4Z of *V. cholerae* and titrated according to the method of Burnet and Stone (1947). It was standardized against purified RDE kindly supplied by Sir Macfarlane Burnet.

Cell suspensions.—Normal fowl erythrocytes were used in 0·5 per cent suspension. Ghost cells for electron-microscopy were prepared according to the technique of Dawson and Elford (1949) and were used in 10 per cent suspension in phosphate buffered saline, pH 6·8. The concentration of cells was calibrated by a photo-electric densitometer.

Calcium chloride-acetate buffer saline (CA saline).—A sodium acetate-acetic acid buffered saline, pH 6·2, containing 0·1 per cent calcium chloride.

Borate buffer.—pH 8·5 (Clark, 1920).

Citrate saline.—Normal saline containing 1 per cent sodium citrate.

Haemagglutinin titration (HA).—Serial two-fold dilutions of the virus suspension were made in normal saline in 0·25 ml. volumes and an equal volume of 0·5 per cent fowl red cell suspension was added to each dilution. The cells were allowed to settle at room temperature and readings were made of the sedimentation patterns. One agglutinating dose (A.D.) was taken as the highest dilution giving partial agglutination and the titres were expressed as the reciprocal of the initial dilution of virus at the end-point.

Haemagglutination inhibition test.—Serial two-fold dilutions of serum were made in normal saline. Normal fowl red cells in 0·5 per cent suspension were added followed immediately by 8 agglutinating doses of test virus. All reagents were used in 0·25 ml. volumes. Readings were made by the sedimentation pattern method, the end-point being taken as partial agglutination. Titres were expressed as the reciprocal of the initial dilution of serum present at the end-point.

Electron-microscopy—All preparations of ghost cells in phosphate buffered saline, pH 6·8 were fixed for 30 min. in 0·1 per cent osmic acid. They were washed in distilled water, spun at 0° for 5 min. at 500 r.p.m. and mounted on collodion films for electron-microscopy. To avoid folding of the ghost membranes during the drying process, mounts were made from very dilute distilled water suspension. Specimens were shadowed at an angle of 15° with a gold-manganese alloy. Electron-micrographs were taken with a Siemens microscope at magnifications of about 5000 using 50 k.v. and a 70μ objective aperture.

Counts were made of the average number of virus particles per ghost cell assuming a random distribution of particles over the surface of the membrane, the area concealed by the nucleus and the unshadowed side of the membrane in contact with the collodion film. Areas to which particle counts related were measured directly from the electron-micrographs using transparent graph paper and the average number of virus particles per ghost cell estimated.

Preparation of virus-coated cells.

Virus-coated cells were prepared according to Burnet's technique (1952) with slight modifications. Instead of packed fowl erythrocytes, the cells were used in 0·5 per cent suspension or, for electron-microscopy, in 10 per cent ghost cell suspension. The reagents were used in the following way : to 40,000 agglutinating doses of treated virus were added 50 ml. of 0·5 per cent red cell suspension or 2·5 ml. of 10 per cent ghost cell suspension. Adsorption was allowed to take place for 1 hr. in a waterbath at 37° with occasional shaking. The cells were centrifuged and the supernatant replaced by 10,000 units RDE in 30 ml. CA saline. After 1 hr. at 37° the cells were spun down, washed once in 1 per cent citrate saline, once in 30 ml. normal saline and re-suspended in 30 ml. normal saline. These treated cells were stable, *i.e.*, were no longer spontaneously agglutinated but were capable of agglutinating normal fowl cells.

Ghost cells were washed and re-suspended in phosphate buffer, pH 6·8, as it was found that cells treated with citrate were not suitable for electron-microscopy. In order to control the amount of virus present on the cells at each stage of these manipulations, particle counts on ghost cells were done and it was shown that approximately 50 to 70 per cent of the virus still remained on the cells after RDE treatment (Fig. 1, 2, 3).

Normal red cells and ghost cells showed no significant differences in their adsorptive activity.

The proportionality between the number of virus particles, as estimated by counts on ghost cells, and the agglutination titre of preparations of influenza virus has recently been demonstrated (Donald and Isaacs, to be published).

RESULTS.

Effects of immune serum on virus-coated cells.

The following experiment was performed in order to show that virus is not removed from RDE-treated cells by specific immune serum.

Of the virus-coated ghost cell suspension 2·5 ml. were centrifuged and the supernatant replaced by 0·5 ml. of specific immune serum diluted 1 in 10. The cells were re-suspended and maintained for 60 min. in a waterbath at 37°. The cells were then spun down, washed, re-suspended in phosphate buffer, pH 6·8 and fixed with osmic acid. Fig. 3 and 4 show electron-micrographs of virus-coated, RDE-treated ghost cells before and after serum treatment. Counts made on several cells of each group showed no significant difference in the number of particles per cell from one group to another.

Antibody absorption with virus-coated cells.

In order to determine the conditions under which antibody could be absorbed most efficiently, the experiments summarized in Table I were carried out.

TABLE I.—*Comparison of Different Methods of Absorbing Antibody with Virus-coated Cells.*

Conditions of absorption.	Antibody unabsorbed. (per cent).
1. One step absorption without re-suspending the cells	33
2. One step absorption, the cells being re-suspended at 15 min. intervals	17
3. One step absorption, the cells being kept in suspension by mechanical shaking	9
4. Three step absorption.	9

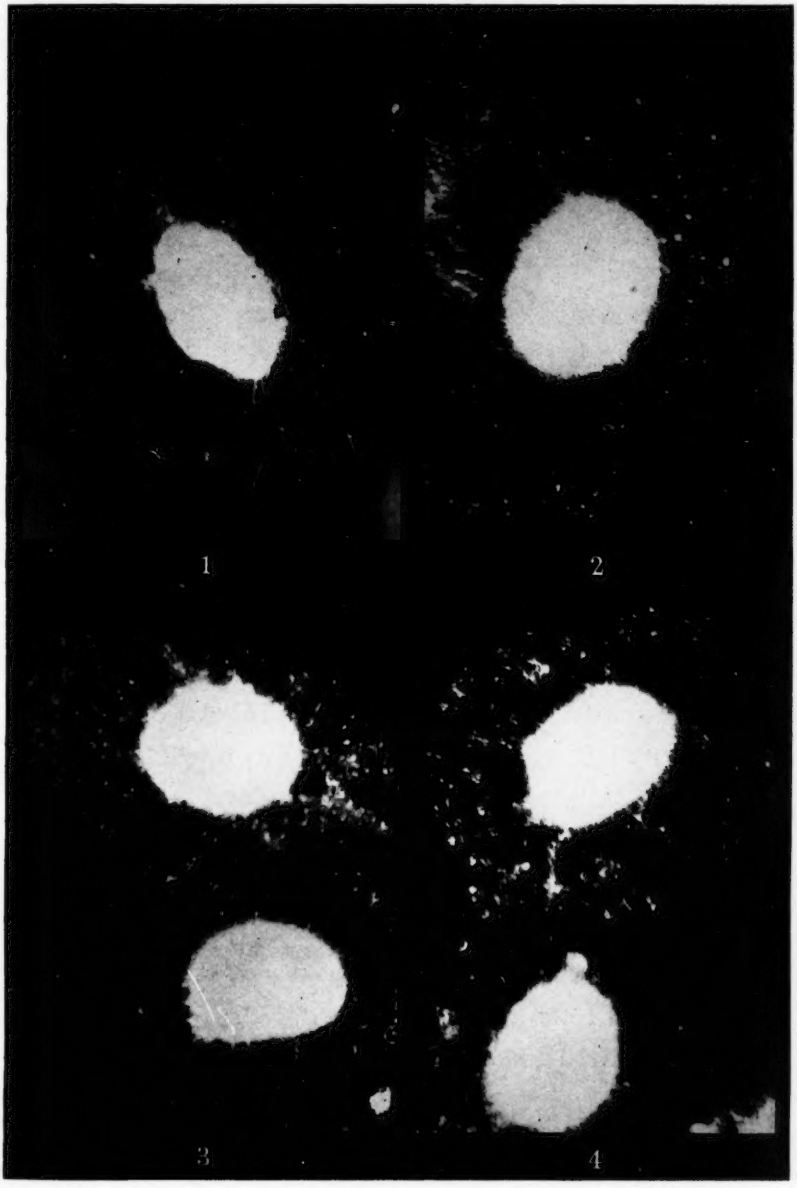
The same amounts of serum and cells were used in each case and were kept in contact for 60 min. at 37°.

The three-step absorption technique was adopted as it proved to be very efficient and the most convenient. Routine absorption tests were done as follows: the final suspension of virus-coated cells was distributed in three centrifuge tubes, spun down and the supernatants discarded. Of the serum to be tested, 1·0 ml. of a 1/20 dilution was added to the first tube, transferred to the second and finally to the third. The tubes were maintained in a waterbath at 37° with occasional shaking to keep the cells in suspension and absorption was allowed to take place for a total of 60 min., *i.e.*, 15 min. for each of the first two steps and 30 min. for the last one.

It was found that unless the virus-coated cells were washed several times before use for absorption some free virus remained in the cell suspension which inter-

DESCRIPTION OF PLATE.

FIG. 1.—Electron-micrograph of a normal fowl erythrocyte.
FIG. 2.—Influenza B virus (Crawley) adsorbed on normal fowl erythrocyte.
FIG. 3.—As in Fig. 2, after RDE treatment.
FIG. 4.—As in Fig. 3, after serum treatment.



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fered with the haemagglutination inhibition test when heterologous absorptions were carried out. As these several washings required extra time and work, it was more convenient to add a drop of packed fowl erythrocytes to the absorbed sera in order to adsorb out any free virus that might be present. After contact for 15 min. at room temperature the cells were centrifuged and the absorbed sera tested for their haemagglutination inhibition titres.

Cross absorption tests.

The technique was first applied to two antigenically unrelated strains of influenza virus, A/PR8, and B/Crawley. The method proved to be specific, giving complete homologous and no heterologous antibody absorption. The specificity of the method was further tested by absorbing a mixture of equal volumes of PR8 and Crawley antisera. One lot of serum was absorbed with PR8 and the other with Crawley-coated cells. The results obtained in this experiment are given in Table II.

TABLE II.—*Absorption of a Mixture of PR8 and Crawley Antisera with PR8- or Crawley-coated Cells.*

Tested with 8 A.D. of—	H.I. titre of serum mixture absorbed with—		
	Normal fowl RBC.	PR8-coated RBC.	Crawley-coated RBC.
A/PR8 . . .	2560	<20	2560
B/Crawley . .	640	640	<20

In Table III results are given of a complete cross absorption test between A/PR8 and a recently isolated A strain, A/England/1/51.

TABLE III.—*Cross Absorption Experiment with A/England/1/51 and PR8 Strains.*

Tested with 8 A.D. of—	PR8 serum absorbed with—			A/England/1/51 serum absorbed with—		
	Normal RBC.	PR8-coated RBC.	A/England/1/51-coated RBC.	Normal RBC.	PR8-coated RBC.	A/England/1/51-coated RBC.
A/PR8 . . .	2560	<20	2560 .	20	—	—
A/England/1/51 .	<20	—	— .	1280	1280	<20

Table IV shows results obtained with two recently isolated strains, A/England/1/51 and A/Missouri/302/53.

TABLE IV.—*Cross Absorption Experiment with A/England/1/51 and A/Missouri/302/53 Strains.*

Tested with 8 A.D. of—	A/England/1/51 serum absorbed with—			A/Missouri/302/53 serum absorbed with—		
	Normal RBC.	A/England/1/51-coated RBC.	A/Missouri/302/53-coated RBC.	Normal RBC.	A/England/1/51-coated RBC.	A/Missouri/302/53-coated RBC.
A/England/1/51 .	1280	<20	1280 .	20	—	—
A/Missouri/302/53 .	60	—	— .	640	640	<20

The results shown in Tables II, III and IV furnish evidence for the specificity of the method.

DISCUSSION.

The influenza antibody absorption technique which has been devised offers several advantages over the methods previously described. It is less time-consuming than the earlier techniques and dispenses with high-speed centrifugation and ultrafiltration. So far as can be calculated from the data of Friedewald (1944) and Hirst (1952), it would appear that considerably less virus is required for absorbing sera by the present method. This suggests that virus adsorbed on red cells may bind antibody more efficiently than free virus, a conclusion similar to that reached earlier by Isaacs and Stone (1949). These authors found that influenza virus combined with antibody more readily in the presence of red cells which the virus could agglutinate than in the presence of red cells which it was unable to agglutinate. It is not yet known why virus bound to the red cell surface should absorb antibody more efficiently than free virus, but if the principle is general it might prove useful in preparing absorbed sera against other viruses.

SUMMARY.

A technique for influenza antibody absorption has been described.

Virus particles adsorbed on fowl erythrocytes in such a way that they cannot readily be removed will absorb antibody.

The method offers several advantages over previous techniques and should prove useful in the study of antigenic relationships among strains of influenza virus.

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EXOPHTHALMOS IN CORTISONE-TREATED EXPERIMENTAL ANIMALS.

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DURING an investigation into the influence of cortisone on the healing of experimental gastric ulcers in guinea-pigs, it was observed that all the test animals developed some degree of exophthalmos (Fig. 1 and 2).

EXPERIMENTAL.

Experiment 1.—Twelve test and 12 control animals were operated upon after they had been anaesthetised with ether. The animals were young adult females, about 300 g. in weight. An ulcer 4 mm. in diameter was produced by cauterizing the gastric mucosa after opening the stomach. Immediately after operation the test animals were given an intramuscular injection of cortisone acetate (" Cortone "—Merck), 5 mg./100 g. body wt., while the controls received the same volume of cortisone-suspending medium. These injections were repeated once daily for 15 days. All animals received a normal mixed diet and plenty of water to drink. Each animal was weighed daily.

All the animals which received cortisone developed exophthalmos. Both eyes were prominent but sometimes one globe was a little more prominent than the other. The degree of exophthalmos was determined by measuring the inter-corneal distance (I.C.D.) with a pair of dividers. For this purpose, surgical anaesthesia was induced with ether, and three readings quickly taken as soon as there was muscle relaxation and absence of the corneal and conjunctival reflexes.

In 15 days the average I.C.D. increased in the cortisone-treated animals by 2·0 mm., whilst the average increase in the control animals was 0·2 mm. The average weight of the test animals increased by 41 g. and that of the controls by 14 g.

The same operation was performed in young adult female rabbits and rats, and exophthalmos was observed in those animals which were injected with cortisone.

Experiment 2.—Each of 6 test rabbits (average wt. approximately 1700 g.) was given a daily intramuscular injection of cortisone (1·0 mg./100 g. body wt.) for 15 days, beginning immediately after operation. Six control rabbits were injected daily with cortisone vehicle.

Experiment 3.—Ten test rats (average wt. approximately 160 g.) were given a daily intramuscular injection of cortisone for 15 days (1·5 mg./100 g. body wt.), beginning immediately after operation. Ten control rats were injected with cortisone vehicle only.

The results of these experiments are given in the Table.

A second series of investigations (Expts. 4, 5 and 6) was undertaken to determine what effect cortisone might have on the eyes of normal guinea-pigs, rabbits and rats. This second series was exactly like the first except that only 6 test guinea-pigs and 6 test rats were used. The results are given in the Table.

In all six experiments there was a highly significant difference in the I.C.D. of the cortisone-treated animals on the 15th day as compared with that of the control animals. In analysing the changes in I.C.D. account was taken of differences in weight change. The mean changes in I.C.D. for the test and control groups were adjusted to correspond to the mean weight change for the whole experiment and the differences in the adjusted means tested to see whether the treatment caused exophthalmos. The effect was significant in every case except in Exp. 2, where the control group showed large weight losses and where the results were consistent statistically with changes in I.C.D. being affected only by changes in weight. In all the other experiments the change in I.C.D. in cortisone-treated animals was disproportionate to the body weight.

TABLE.—*Mean Wt. Change in the Whole Experiment, Mean Changes in I.C.D. in the Two Groups, adjusted for Wt. Changes, with Standard Errors of the Differences.*

Experiment.	Mean wt. change.	Mean change in I.C.D.			Significance.
		Test.	Control.	Difference.	
1	28	1·56	0·61	0·95±0·31	+ +
2	−59	1·78	0·88	0·90±0·90	N.S.
3	−8	1·58	0·07	1·51±0·44	+ +
4	31	2·37	−0·20	2·57±0·32	+ + +
5	43	4·57	0·35	4·22±0·92	+ +
6	10	2·06	0·02	2·04±0·50	+ +

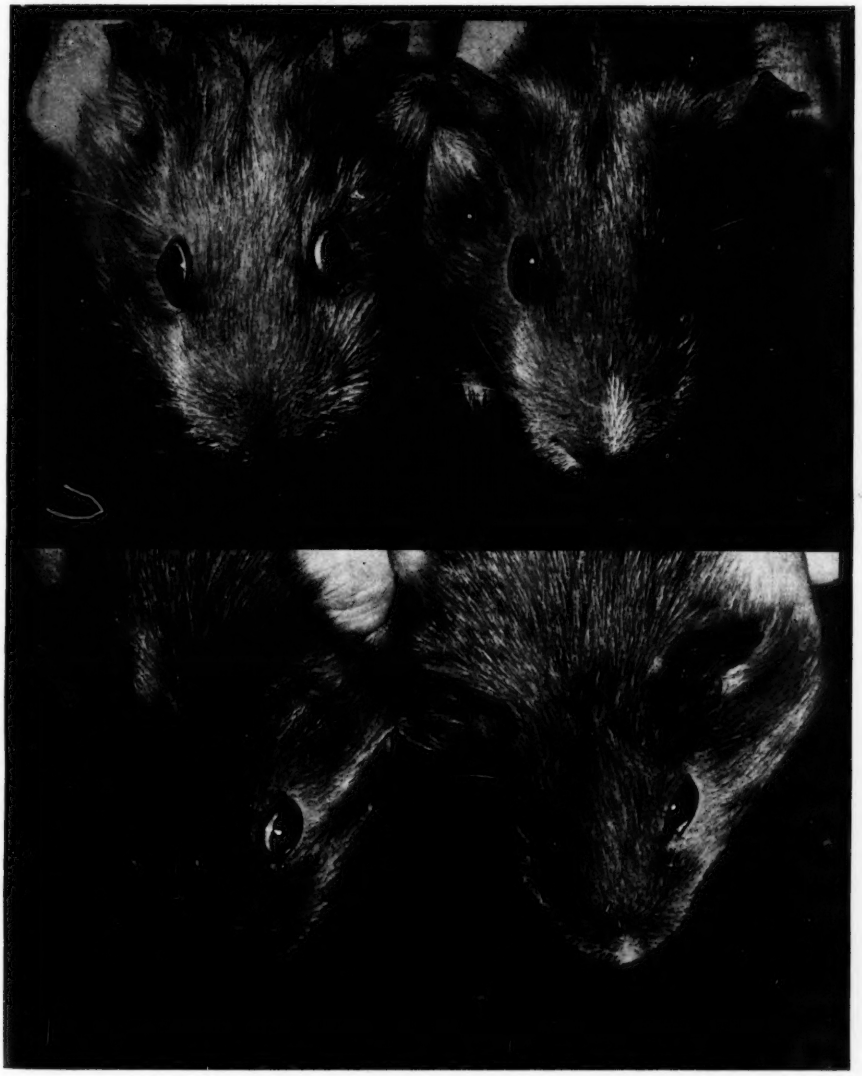
N.S. Not significant.
\+ Significant at the 5 per cent level.
\+ + Significant at the 1 per cent level.
\+ + + Significant at the 0·1 per cent level.

In a seventh experiment, the effect of thyroidectomy followed by the successive and separate administration of cortisone and desiccated thyroid on the eyes of guinea-pigs was studied.

Experiment 7.—Mature adult female animals were used. Sixteen were thyroidectomised and these were divided into a test group and a control group, each containing eight animals. All were given a normal mixed diet and plenty of drinking water. Their weight and I.C.D. were estimated at intervals of 15 days. The first weighings and measurements were made immediately before thyroidectomy, on the first day of the experiment. On the 15th day, after the second series of readings were taken, the test animals were given an intramuscular injection of cortisone acetate (5 mg./100 g. body wt.) while the control animals received an equal volume intramuscularly of cortisone vehicle. The injections

DESCRIPTION OF PLATE.

Two views of the same guinea-pigs on the 15th day after operation (production of a gastric ulcer after laparotomy). The animal on the left was given daily intramuscular injections of cortisone acetate (Merck), 5·0 mg./100 g. body wt., while the control animal on the right was given daily intramuscular injections of cortisone vehicle only.



were repeated daily for 15 days. From the 30th day the test animals were given desiccated thyroid (8 mg. orally) every other day. Final readings were taken on the 45th day of the experiment.

After thyroidectomy, the I.C.D. of each group of animals increased by an average of 1·35 mm. while the weight decreased (average of 10 g. in the " test " animals and of 20 g. in the " control " animals).

In the second 15-day period, neither group showed any change in I.C.D., but the cortisone-treated group gained some weight (average of 18 g.) while the control group lost some weight (average of 5·5 g.).

In the third 15-day period, the average weight of the thyroid-treated animals showed a sharp fall of 40 g. and the average I.C.D. decreased by 1·0 mm. The average weight and I.C.D. of the control animals remained unaltered. Thus, thyroidectomy alone produced exophthalmos which was unaltered by cortisone and diminished by desiccated thyroid gland. Statistical analysis showed that the effect of the desiccated thyroid gland on the I.C.D. could be correlated with the decline in the body weight.

DISCUSSION.

There is a remarkable difference of opinion regarding the pathogenesis and treatment of exophthalmos. Even in such a comparatively simple and fundamental problem as the existence or non-existence of exophthalmos following thyroidectomy in animals there is a difference of opinion. Vincent and Jolly (1904), Gley (1910) and Dobyns (1945) have described such exophthalmos whereas Lambie (1939) and Aird (1940) failed to find any. The cause or causes of such a discrepancy is not always clear; but in any investigation concerning exophthalmos in animals it is important to measure the position of the eyes and also to consider the influence of change in body weight in relation to the eyes (Pochin, 1944).

Recently, different opinions have been expressed on the effects of cortisone and ACTH on the position of the eyes of animals. Aterman (1951, 1952) produced exophthalmos by giving daily injections of cortisone (approximately 6 mg./100 g. body wt.) to normal and thyroidectomised guinea-pigs, whereas Smelser and Ozanics (1951) failed to produce exophthalmos in thyroidectomised guinea-pigs after giving injections of cortisone (approximately 2 mg./100 g. body wt.) or ACTH (approximately 2 mg./100 g. body wt.). Jeffries (1949) also obtained a negative result in guinea-pigs using daily injections of highly purified ACTH (0·5 to 1·5 mg. daily) for 48 hours only. Recently Aterman and Greenberg (1953) have reported the occurrence of exophthalmos in cortisone-treated rats.

There seems to be general agreement that cortisone or ACTH suppresses thyroid function (Hill, Reiss, Forsham & Thorn, 1950; Money, Kraintz, Fager, Kirschner & Rawson, 1951; Wolfson *et al.*, 1951; Frederickson, Forsham and Thorn, 1952; Kuhl and Ziff, 1952). Much of this opinion is based on a study of the uptake of I^{131} by the thyroid. Some investigators (Hill *et al.*, 1950; Frederickson *et al.*, 1952) believe that this thyroid inhibition arises from a suppression of thyroid-stimulating hormone (T.S.H.), but Epstein, Cantarow, Friedler and Paschkis (1953) have demonstrated that cortisone inhibits the thyroid-stimulating action of T.S.H. at extrapituitary level. If cortisone inhibits

thyroid function, it is perhaps not surprising that it does not increase the exophthalmos following thyroidectomy.

SUMMARY.

Experiments are described in which exophthalmos developed in young adult guinea-pigs, rabbits and rats following the daily intramuscular injection of cortisone acetate.

In another experiment exophthalmos was produced in mature adult guinea-pigs by thyroidectomy. Cortisone failed to increase this exophthalmos. Desiccated thyroid gland decreased it.

I am indebted to Professor J. S. Young for advice and guidance, to many assistants for much technical help, to Dr. F .H. C. Marriott who made the statistical analysis, and to the Medical Research Council for supplies of cortisone.

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THE INHIBITION OF A WATER DIURESIS BY SHORT PERIODS OF MUSCLE ISCHAEMIA.

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IN addition to the many reports on the fall in urine output after injury in man (Minami, 1923; Bywaters and Beall, 1941; Bywaters, 1945; Darmady, 1948, and others) a decrease in the rate of urine flow has been reported after occlusion of the circulation to the hind limbs in rabbits (Bywaters and Popják, 1942), cats (Eggleton, 1944; Cort, 1951), dogs (Green, Dworkin, Antos and Bergeron, 1944) and rats (Green and Stoner, 1950). The mechanism of this effect has been studied in some detail and the importance of such factors as fluid loss into the damaged limb (Koletsky and Gustafson, 1950), reduction in renal blood flow (Eggleton, 1944; Cort, 1951) and alterations in the pattern of renal blood flow (Trueta, Barclay, Daniel, Franklin and Pritchard, 1947) has been emphasized. In most of these experiments the periods of ischaemia were sufficient to produce serious shock. This is not necessary, however, for we have previously shown (Green and Stoner, 1950) that short and apparently innocuous periods of limb ischaemia have an antidiuretic effect in the rat and that, under these conditions, an antidiuretic substance appears in the urine. This paper presents the results of the further investigation of this effect which show that the antidiuretic hormone of the posterior lobe of the pituitary is an additional factor in the antidiuretic response to injury.

METHODS.

Male albino rats (180–200 g.) from one source were used and fed on Edinburgh rat cube No. 86 (20 per cent protein) given *ad lib.* Each rat was used in one experiment only. An adequate number of controls, closely matched by weight with the experimental animals was always used (Gaunt, Liling and Cordsen, 1945). To ensure adequate and equal hydration the rats were given 5·0 ml. water/100 g. body wt. at 37° by stomach tube at about 5 p.m. on the day before the experiment and allowed free access to water, but not food, overnight. For the experiment the rats were water-loaded with 3 doses of 5·0 ml. water/100 g. body wt. at 37° given by stomach tube at hourly intervals. The details of the diuresis experiments will be given in another paper (see also Dexter and Stoner, 1952) as will various operative details and the methods for determining aortic blood pressure, glomerular filtration rate (GFR), blood distribution in the kidney, water absorption, the specific gravity and Cl' concentration of the urine and the adenyl compounds of the plasma and urine extracts. Urine extracts were prepared from Seitz-filtered urine by the methods of Grollman and Woods (1949) and Taylor and Walker (1951).

Limb ischaemia was produced with rubber tourniquets (Rosenthal, 1943). A 2 hr. period of bilateral hind limb ischaemia was used routinely and at an environmental temperature of 19–21° this was never fatal. The amount of fluid lost into the limb after release of the tourniquet was determined by water displacement.

Ferritin (VDM) in plasma was determined by the method of Zweifach, Chambers, Lee and Hyman (1948). In some experiments the vaso-excitor material (VEM) was removed

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beforehand by incubating the plasma with rat kidney slices for 1 hr. at 37° aerobically (Shorr and Zweifach, 1948).

RESULTS.

Effect of hind limb ischaemia on a water diuresis.

Little change in the rate of urine flow occurred during the period of ischaemia. When bilateral hind limb tourniquets were applied under ether anaesthesia at the time of the third dose of water there was a short inhibition of the diuresis but after an hour, urine output was not significantly different from that in the controls (Fig. 1).

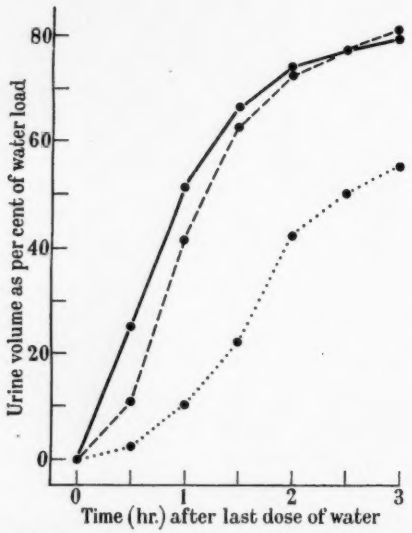

FIG. 1.—The effect of the application and release of bilateral hind limb tourniquets on water diuresis in rats. Each point represents the mean of 5 rats.

●——● Controls.

● - - - - ● Rats with tourniquets applied at 0 time. Only the point at 0·5 hr. is significantly different from the controls ($P < 0.05$).

● . . . . ● Rats in which the tourniquets were released at 0 time after being in position for 2 hr. All points significantly different from those on control curve; $P < 0.05$ at 0·5 and 3·0 hr. and < 0.01 at 1·0, 1·5, 2 and 2·5 hr.

When the tourniquets were removed at the time of the last dose of water, after being in position for 2 hr., more definite changes were seen (Fig. 1). Bilateral hind limb ischaemia had a greater effect than unilateral ischaemia (Fig. 5), and gave an immediate antidiuretic effect lasting about 90 min. The rate of urine flow then increased to equal that in the normal rats but even after 24 hr. the whole of the excess water had not been excreted. This latter may be related to the prolonged increase in the water content of the damaged tissue (Stoner and Green, 1948). Certainly water intoxication (Gaunt, 1944) was not seen in these rats although it occurred in 9 of 13 similar experiments on adrenal medullecto-

mized rats. Medullectomized rats show a poor response to water loading (Dexter and Stoner, 1952) and their antidiuretic response to limb ischaemia was correspondingly enhanced (Fig. 2). The antidiuresis was accompanied by a rise in the specific gravity of the urine and by a reduction in the urinary Cl′ concentration and excretion (Table I). This change in Cl′ excretion is probably due to Cl′ accumulation in the damaged tissue (Fuhrman and Crismon, 1951). Apart from this the picture closely resembles that produced in these rats by physio-

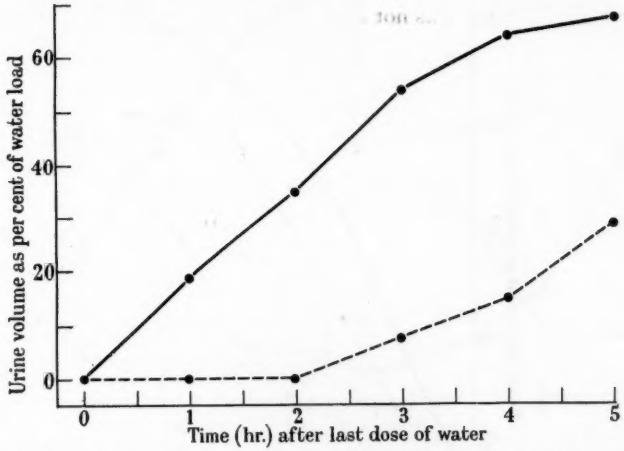

FIG. 2.—The effect of the release, at 0 time, of bilateral hind limb tourniquets which had been in position for 2 hr., on a water diuresis in medullectomized rats 40 days after operation. Each point represents the mean of 5 rats and all the points on the experimental curve are significantly different ($P < 0.01$) from the controls.
●——● Medullectomized controls.
● - - - - ● Medullectomized rats after limb ischaemia.

logical doses of " pitressin ". A further point of similarity was in the response to cortisone acetate (Merck) which antagonized the antidiuretic response when given 2–3 hr. before either the injection of " pitressin " (Dexter *et al.*, 1953) or the release of the tourniquets (Fig. 3).

TABLE I.—*Effect of Release of Bilateral Hind Limb Tourniquets, in place for 2 hr., on Sp. G. and Cl′ Content of the Urine of Diuretic Rats.*

		Controls.		Experimental.	
	Hour.	Sp. g.	Total Cl′ excretion mg./100 g./hr.	Sp. g.	Total Cl′ excretion mg./100 g./hr.
Ischaemic period	1st	1·008±0·003	0·81±0·16	1·009±0·004	0·76±0·19
	2nd	1·002±0·001		1·002±0·001	
Post-ischaemic period.	1st	1·002±0·001	—	1·012±0·004*	—
	2nd	1·003±0·001	0·86±0·21	1·009±0·003*	0·29±0·10*
	3rd	1·006±0·002	—	1·004±0·002	—

* Significantly different from controls at $P < 0.01$. Each figure represents the mean ± S.D. of 5 rats.

Water absorption after limb ischaemia.

The rate of water absorption after the release of bilateral hind limb tourniquets applied for 2 hr. is shown in Fig. 4. The application of the tourniquets under ether anaesthesia did not affect absorption as the volume of unabsorbed water at the time of release was the same in both groups. Water absorption was, however, significantly delayed during the first hour after release mainly

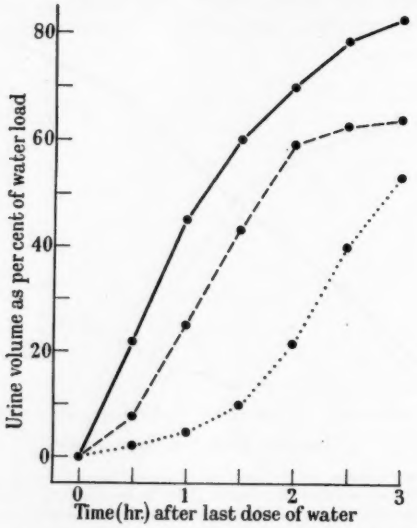

FIG. 3.—The effect of cortisone on the antidiuretic action of limb ischaemia.

● ——— ● Controls showing normal water diuresis.
● . . . . ● Rats in which the tourniquets were released at 0 time after being in position for 2 hr.
● – – – – ● Rats in which the tourniquets were released at 0 time after being in position for 2 hr., 2·0 mg., cortisone acetate/100 g. body wt. given intramuscularly at the beginning of the period of ischaemia.

Each point represents the mean of 5 rats. The points on the interrupted line are significantly different at $P < 0.01$ from those on the dotted line at 1·5 and 2·0 hr. and at $P < 0.05$ at 1·0 and 2·5 hr.

because of slow gastric emptying. Under similar conditions Whiteley and Green (1952) have found radiological evidence of reduced gastric motility. The volume of water absorbed but not excreted was, however, the same in the two groups at the time of release of the tourniquets and during the next hour. Thus, the immediate reduction in urine flow was not due to delayed absorption of water but to failure to excrete the water already absorbed.

Fluid loss into the limb after ischaemia.

The increase in volume of the hind limbs one hour after a 2 hr. period of ischaemia was 0.71 ± 0.33 ml./100 g. body wt. after correcting for post-mortem contraction (Green *et al.*, 1944). As the volume of urine passed in the first hour

after such a period of ischaemia was 2·8 ± 0·4 ml./100 g. body wt. less than in the controls the effect could not be completely explained by loss of fluid into the damaged part.

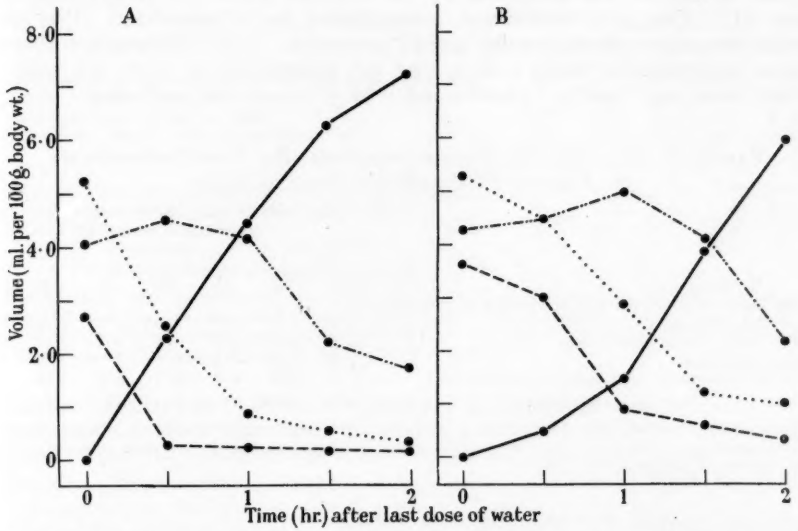

FIG. 4.—Water absorption and urine excretion in normal rats (A) and rats after the release at 0 time of bilateral hind limb tourniquets which had been in place for 2 hr. (B). The observations were made after 3 doses of water, each 5·0 ml./100 g. body wt., had been given at hourly intervals. The volume of water absorbed but not excreted has been calculated. Each point represents the mean of 4 rats.

●————● Urine volume. ● - - - - ● Unabsorbed water in stomach.
●— - - —● Water absorbed, but not excreted. ● . . . . ● Unabsorbed water in intestine.

Values of t (obtained by comparison with the means of control animals).

Time (min.)	0.	30.	60.	90.	120.
Urine volume	—	2·24	3·09	3·17	2·13
Water absorbed but not excreted	0·16	0·54	1·17	2·53†	1·25
Water in stomach	1·40	4·61*	1·98	0·95	0·62
Water in intestine	0·21	2·70†	2·83†	1·05	0·78

* $P < 0·01$. † $P < 0·05$.

Renal circulation after limb ischaemia.

Our interest here was to see if the antidiuretic effect of this short, non-fatal period of limb ischaemia could be explained by changes in the renal circulation. Naked-eye and histological examination showed no evidence of alteration in the pattern or decrease in the renal blood flow. This was confirmed by Berlin Blue and India ink injections. The aortic blood pressure remained within the normal range after a 2 hr. period of bilateral hind limb ischaemia and there was no alteration in the GFR (creatinine clearance). In 24 controls this latter was 0·67 ± 0·10 ml. plasma/100 g. body wt./min. and in 10 rats examined immediately after a 2 hr. period of bilateral hind limb ischaemia it was 0·65 ± 0·15 ml. plasma/100 g. body wt./min.

Antidiuretic activity of plasma after limb ischaemia.

Plasma obtained from rats 30 min. after a 2 hr. period of ischaemia showed moderate antidiuretic activity when injected intraperitoneally into water-loaded rats and this effect was prevented by the addition of 0·01 M thioglycollic acid (Table II). The antidiuresis was accompanied by a significant ($P < 0·01$) increase in urinary specific gravity and Cl′ excretion. The GFR was not affected in these experiments, being $0·67 \pm 0·01$ ml. plasma/100 g. body wt./min. in the rats receiving "active" plasma and $0·64 \pm 0·14$ in the controls.

TABLE II.—*The Effect of Plasma from Rats after Limb Ischaemia on the Diuresis Produced by 3 Doses of Water.*

Treatment.	Urine output (ml./100 g. body wt.), after last dose of water, at (min.).						
	15.	30.	45.	60.	90.	120.	180.
Plasma taken 30 min. after 2 hr. period of bilateral hind limb ischaemia	0·6*	1·3*	2·3*	3·5*	5·2†	6·2†	8·7
Same + 0·01 M thioglycollic acid	1·3	2·6	4·5	6·8	8·1	8·9	9·2
Normal rat plasma	1·3	2·5	4·3	6·8	7·9	8·8	9·2
Equivalent volume 0·9 per cent NaCl (Controls)	1·2	2·6	4·4	6·7	8·0	9·0	9·4

* Significantly different from controls at $P < 0·01$. † at $P < 0·05$.

Three doses of water, each 5·0 ml./100 g. body wt., given at hourly intervals. Plasma injected intraperitoneally in doses of 1·0 ml./100 g. body wt. at the same time as the last dose of water. Each figure represents the mean of 5 rats.

Antidiuretic activity of urine after limb ischaemia.

Extracts of urine, collected after release of the tourniquets, prepared according to the methods of Grollman and Woods (1949) and Taylor and Walker (1951) contained an antidiuretic substance (Table III) with the following properties. It significantly ($P < 0·01$) increased the specific gravity and Cl′ concentration of the urine but not the amount of Cl′ excreted. The absence of increased Cl′ excretion after the injection of urine extracts has been noted by Ham and Landis (1942). This antidiuretic substance was inactivated both by the addition of 0·01 M thioglycollic acid and by exposure to 2·0 N NaOH for 1 hr. at 20° (Table III). Neither antidiuretic extract altered the GFR. This was $0·63 \pm 0·12$ ml. plasma/100 g. body wt./min. in rats given Grollman and Wood's extract, $0·64 \pm 0·09$ in those given Taylor and Walker's extract and $0·67 \pm 0·10$ in the controls.

Although the antidiuretic substances in the plasma and urine after limb ischaemia behave very like the antidiuretic hormone of the posterior lobe of the pituitary the possibility that their activity is due to adenyl compounds must be examined since these compounds, especially adenosine triphosphate (ATP), exert a powerful antidiuretic effect (Dexter *et al.*, 1953) and since these compounds are increased in the blood of certain species after limb ischaemia (Stoner and Green, 1944). In the present experiments this explanation is unlikely as active adenyl compounds have not been demonstrated in the blood stream of the rat even after longer periods of limb ischaemia (Green and Stoner, 1950). The U.V. absorption, between 230–290 mμ., of perchloric acid extracts of plasma taken 1 hr. after a 2 hr. period of bilateral hind limb ischaemia was increased when compared with that of plasma taken before the tourniquets were applied. However, the increase was not similar to that produced by the addition of adenosine

TABLE III.—*Effect of Extracts prepared from the Pooled Urine of 6 Normal Diuretic Rats and 6 Rats after a 2 Hr. Period of Bilateral Hind Limb Ischaemia.*

Treatment.	Urine output (ml./100 g. body wt.), after last dose of water, at (min.). 30.	60.	90.	120.	180.
A.					
Extract from urine passed after hind limb ischaemia	0·5*	2·0*	4·4†	6·0†	8·6
Same + 0·01 M thioglycollic acid	2·2	4·3	6·3	8·5	9·0
Extract from normal rat urine	2·4	4·5	6·5	8·7	9·3
Controls. Equiv. vol. 0·9 per cent NaCl	2·2	4·5	6·6	8·8	9·3
B.					
Extract from urine passed after hind limb ischaemia	1·0*	2·1*	4·3†	6·5†	8·8
Same treated with 2·0 N NaOH for 1 hr. at 20°	2·4	4·8	7·0	8·6	9·1
Extract from normal urine	2·6	5·1	7·2	9·0	9·3
Controls. Equiv. vol. 0·9 per cent NaCl	2·5	5·0	6·9	8·8	9·2

* Significantly different from controls at $P < 0{\cdot}01$. † at $P < 0{\cdot}05$.

The extracts were prepared by the methods of Grollman and Woods (A) and of Taylor and Walker (B), and the urine collected over a 3 hr. period. The extracts, in 0·9 per cent NaCl, were injected intraperitoneally at the same time as the last dose of water. The figures represent the mean of 6 rats, each receiving approximately ⅛th of the total extract.

or inosine or mixtures of these to plasma. A similar increase was seen in the control animals after ether anaesthesia and cardiac puncture. There was no increase in the absorption of the urine extracts at these wavelengths after limb ischaemia. The maximum amount of ATP in these extracts, assuming that all the absorption at 260 mμ. was due to this compound, could only have been 222 μg./ml. of the solution injected which was much too low to account for their antidiuretic activity.

Another substance which appears in the blood stream after limb ischaemia and which has an antidiuretic action is ferritin (Shorr, Zweifach, Furchgott and Baez, 1951; Baez, Mazur and Shorr, 1952). Ferritin was readily detected in all specimens of plasma taken 3 hr. after a 5 hr. period of bilateral hind limb ischaemia but none was found at the same interval after a 2 hr. period of ischaemia, even after the destruction of any VEM which might be present.

Effect of hind limb denervation on the antidiuretic response to hind limb ischaemia.

It was found that when tourniquets were applied within the anaesthetic area produced by hind limb denervation neither the slight response to their application nor the much greater antidiuretic effect produced by their release occurred (Fig. 5).

DISCUSSION.

It has been shown that when limb ischaemia is produced in the water-loaded rat by tourniquets there is a brief inhibition of urine flow on their application and a much greater antidiuretic effect on their release. From our results it is possible to form a picture of the mechanism of the antidiuresis following the release of tourniquets which have been in position for a short time.

The response is very similar to that produced by physiological doses of "pitressin" both in the form of the excretion curve and the response to cortisone. The result of this short period of ischaemia cannot be attributed to a fall in blood

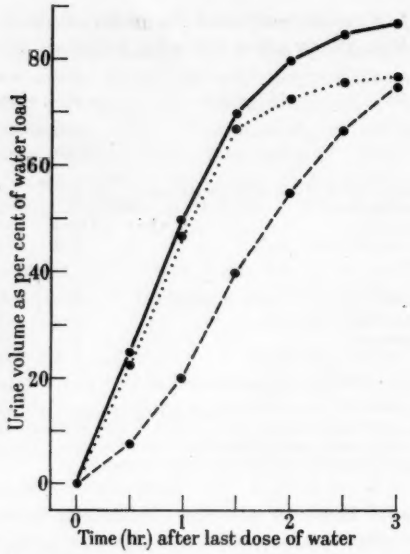

FIG. 5.—The effect of hind limb denervation on the antidiuretic effect of the release, at 0 time, of a unilateral hind limb tourniquet which had been applied for 2 hr. Each point represents the mean of 5 rats.

● ———— ● Controls.

● . . . . ● Tourniquet removed from denervated limb. No significant difference from controls.

● – – – – ● Tourniquet removed from normal limb. Significantly different from controls ($P < 0.05$) up to 2 hr.

pressure or GFR nor to delayed water absorption. Although the rate of water absorption from the gut was reduced by limb ischaemia the water load (water absorbed but not excreted) was always greater than in the controls. The fluid lost into the damaged tissue is insufficient to account for the whole effect. It is difficult to say how much this exudate contributes to the effect. Although the total extracellular space of the body is unaltered by limb ischaemia (Holmes and Painter, 1947), the fluid of the exudate is probably not available for other purposes. Mere resemblance to the effect of " pitressin " does not prove that the antidiuretic effect of ischaemia is due to posterior pituitary hormone (Verney, 1946), but the possibility becomes more probable with the demonstration of an antidiuretic substance in the plasma and urine with properties similar to " pitressin," *e.g.*, inactivation by thioglycollic acid (Van Dyke, Chow, Greep and Rothen, 1942) and NaOH (' British Pharmacopoeia,' 1948). Under the conditions of our experiments the activity of the plasma and urine could not have been due to ferritin or adenyl compounds. Even if these compounds had been involved, posterior pituitary hormone would still be the effective antidiuretic agent since it is liberated by these substances (Baez *et al.*, 1952 ; Dexter *et al.*, 1953). Our conclusion is that the antidiuresis observed after removal of the tourniquets is due to the release of antidiuretic hormone from the posterior lobe of the pituitary and the experiments on denervated limbs indicate that the main factor concerned

in this release is the flow of afferent nervous impulses from the damaged limb. That such impulses produce antidiuresis in this way has been shown, in dogs, by Rydin and Verney (1938) and Verney (1947). Rothballer (1953) has also shown that such stimuli reduce the amount of neurosecretory material in the neurohypophysis of the rat.

As cortisone inhibits the effect of " pitressin " (Dexter *et al.*, 1953) it might be wondered why antidiuresis is seen after limb ischaemia which itself stimulates adrenal cortical secretion (Popják, 1944 ; Stoner and Green, 1950). Adrenal medullectomy prevents the early phase of this adrenal cortical secretion (unpublished results) and the greater antidiuretic effect of limb ischaemia after this operation is probably a measure of the antagonism occurring in the normal animal.

The above considerations apply to short periods of ischaemia unaccompanied by gross general disturbance. How far they account for the much greater decrease in the rate of urine flow after more prolonged ischaemia is not known. Other factors such as the fall in blood pressure and GFR (Eggleton, Richardson, Schild and Winton, 1943) and greater local fluid loss will play a part in this effect, but even so the liberation of posterior pituitary hormone, in response to afferent nervous stimuli, must be concerned in the early stages. It may even play a part in the latter stages when ferritin appears in the blood .

The antidiuresis accompanying the application of the tourniquets has not been studied in detail. As it is also prevented by denervation of the limb its mechanism is probably similar to that which follows their release.

SUMMARY.

When short periods of hind limb ischaemia are produced in rats by tourniquets a brief inhibition of a water diuresis occurs when they are applied and a much greater one when they are released.

The changes following release are thought to be due to the liberation of antidiuretic hormone from the posterior lobe of the pituitary as a result of afferent nervous stimuli from the damaged area.

The relationship of this to the more severe disturbance of renal function after longer periods of limb ischaemia is discussed.

The expenses of this work were defrayed by the Medical Research Council. During the course of this work one of us (D. D.) held a Medical Research Council Studentship. Our thanks are due to the Medical Research Council Committee on ACTH, Cortisone and Related Substances for supplies of cortisone acetate and to Mr. C. J. Threlfall for his advice on chemical matters.

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SOME OBSERVATIONS ON THE EFFECT OF X-RAYS ON THE BEHAVIOUR OF A TEMPERATE PHAGE IN TWO STRAINS OF *PS. PYOCYANEA*.

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X-RADIATION has not been as widely used as ultra-violet light in the study of bacteriophage behaviour. Wollman and Lacassagne (1940) used X-rays as a means of determining the size of bacterial viruses and showed that with the phages they tested resistance to irradiation was inversely proportional to size. Later Latarjet (1948), substituting these rays for those of ultra-violet light in the technique devised by Luria and himself (1947), followed the development of phage T2 during its growth in a single growth cycle in its bacterial host. Watson (1950) has also studied the effect of these rays on the phages of the T series.

Some observations on the effects of X-rays on phage growth in two strains of *Pseudomonas pyocyanea* have been made here and a comparison drawn between them and those of ultra-violet light. The effects of the latter have already been extensively studied on these two strains by Benzer and Jacob (1953) and on the sensitive one and another lysogenic strain derived from it by Jacob (1952*a*, *b*).

MATERIALS AND METHODS.

Origin of bacterial and phage strains.

The strains of bacteria and the phage were selected originally by Jacob as a lysogenic system capable of growing in a synthetic medium and so being readily amenable to the study of effect of changes in environment on induction. The temperate bacteriophage P8 was derived from a lysogenic culture of *Ps. pyocyanea* (P.p8 from the Collection of the Pasteur Institute) and could be propagated on the sensitive strain of *Ps. pyocyanea* P13. From this strain could be derived artificial lysogenic cultures carrying phage 8. One of these strains was designated *Ps. pyocyanea* P13(8). These strains were kindly supplied by Dr. F. Jacob for this study.

Media used.

1. Difco broth, to which was added 1 per cent peptone (Vaillant), was used for growing the indicator strain (P13) and with the addition of 1·5 per cent Difco agar for the determination of plaque counts by Gratia's method. It was also used occasionally for irradiation experiments.

2. Yeast casein hydrolysate medium (56 YC). This medium, which was used in the majority of experiments, consisted of 10 per cent yeast extract, 20 per cent casein hydro-

lysate, 10 per cent 56 medium and 60 per cent distilled water. Medium 56 was originally devised by Dr. J. Monod and has the following composition :

KH_2PO_4	13·6 g. (0·1 M)	for pH 7·4.
KOH	approximately 8 ml. of a 10 N solution	
$(NH_4)_2SO_4$	2·0 g.	
$MgSO_4.7H_2O$	0·2 g.	
$CaCl_2$	0·01 g.	
$FeSO_4$	0·0005 g.	
Glucose	2·0 g. (sterilized separately).	
Water	to 1000 ml.	

3. Lactate medium 56 : 0·9 g. ammonium lactate was substituted for the glucose in the synthetic 56 medium above.

X-radiation.

(1) Two X-ray tubes were used :

(*a*) A tube with a molybdenum target, operating at 33 kV. and 15–40 mAmp., with an average λ of 0·9 Å was used for delivering intensities as required of between 3000 and 30,000 R min.$^{-1}$ measured at the surface of the preparations. These preparations, the depths of which were 1–2 mm., were irradiated in open dishes. A correction for the effective radiations within the preparations was made : 715 R for every 1000 R at the surface.

(*b*) For high intensities, a Machlet tube was used operating at 37 kV. and 40 mAmp. and delivering in the preparation an intensity of 214,000 R min.$^{-1}$

(2) A 10 curie cobalt 60 radiation source. This source of radiation from radio-active cobalt was designed by Dr. F. Gilbert and delivered in the preparation an intensity of 445 R falling to 425 R min.$^{-1}$ during the period when it was used in some of the experiments.

No difference was noted in the effects of the soft X-rays from the tubes and those from the cobalt source on the survival curves of the bacterial strains or of free phage. Similarly no quantitative difference between them could be found in their effects on the *Bact. coli* K.12 lysogenic system (Dr. A. Marcovitch, personal communication).

Turbidity measurements were made in a Meunier photometer, one division of the scale corresponding to approximately 2×10^6 bacteria/ml.

Glossary of Terms.

Burst size.—The number of phage particles released by lysis of a bacterium.

Exponential cultures.—Bacterial cultures in the exponential or log phase of growth.

Induction.—The setting in motion of the prophage to phage sequence in lysogenic bacteria.

Infectious centres.—Centres of plaque formation whether produced by free phage or by an induced bacterium.

Luria-Latarjet or L.L. curves.—The curves indicating the resistance to radiations of intra-bacterial phage during its developmental cycle.

Lysogenic bacteria.—Bacteria which perpetuate hereditarily the power of producing bacteriophage. The nature of the carried prophage is noted by including its number in brackets after its host's name, *i.e.*, *Ps. pyocyanea* P13(8).

Mature or infective phages.—Phages which are capable of infecting receptive or sensitive bacteria.

Multiplicity of infection.—The number of phages infecting one bacterium.

Residual growth.—The increase in optical density of a lysogenic culture during the early period following induction.

Temperate phages.—Phages which are capable of giving rise to lysogenic systems.

Vegetative phage.—The non-infective form in which phage multiplies in both induced and in bacteria infected from without.

Virulent phages.—Phages which are not capable of giving rise to lysogenic systems.

Prophage.—The form in which phage is transmitted from one bacterial generation to another in a lysogenic culture.

For further definitions, relative to this subject, see Jacob, Lwoff, Simonovitch and Wollman (1953).

RESULTS.

Resistance of free phage, P8.

Free phage was irradiated in broth, 56 YC, and in 56 medium with 10 per cent broth added. To 56 medium 10 per cent broth was added to avoid the secondary toxic effects due to irradiation of a synthetic medium, as Latarjet and Ephrati (1948) had shown that these could be countered by the addition of amino-acids or broth.

In spite of its relatively large size (80 mμ.) this phage was very resistant to X-rays, irrespective of the medium in which it was suspended (Fig. 1). It was

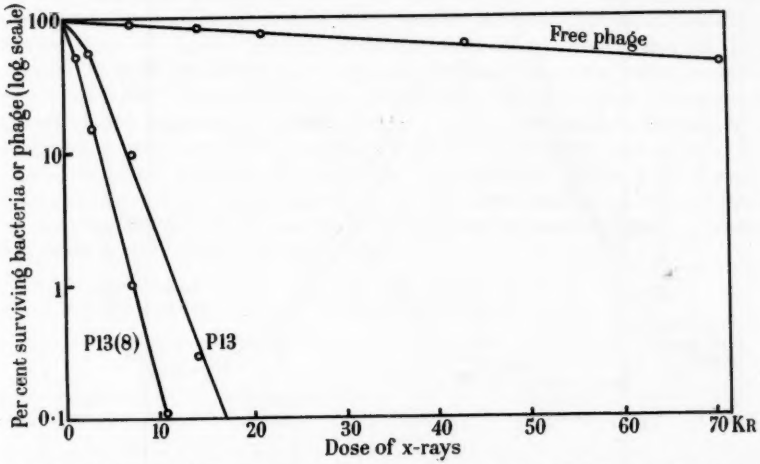


FIG. 1.—Survival curve of *Ps. pyocyanea* P13 and P13(8) and phage P8 following X-radiation. The phage is much more resistant than its bacterial hosts, its inactivation dose being 80,000 R. 56 YC medium.

slightly more sensitive in broth than in 56 YC, the inactivation dose (e^{-1} survival) being 75,000 R in broth and 86,000 R in 56 YC. This temperate phage was thus found to be more resistant to X-rays than the virulent even number T phages. Watson (1950) found the inactivating dose for phage T2 to be only 40,000 R. An analagous situation has been noted with ultra-violet light (Weigle and Delbruck, 1951 ; Jacob, 1952*b*).

Resistance of bacterial strains.

Both strains of *Ps. pyocyanea* were sensitive to X-radiation, the lysogenic P13(8) being more so than P13 (Fig. 1). The increased sensitivity of P13(8) was partly due to killing by induction. With low doses of X-rays up to 1700 R or so bacteria were killed by induction only, but with increasing doses the direct killing effect of the radiations was added as shown by the curve of survival suddenly becoming steeper (Fig. 3). The theoretical survival curve for killing by phage induction alone, calculated from the survival curves of both bacterial strains,

is steeper than the early part of the curve of P13(8). This would suggest that the lysogenic strain besides being killed by induction was also more sensitive to the direct action of X-rays than its original non-lysogenic parent, P13.

*Induction of lysogenic strain P*13(8).

As was first shown by Lwoff, Simonovitch and Kjeldgaard (1950), in certain lysogenic cultures, the majority of bacteria can be induced to produce bacteriophage by irradiating them with ultra-violet light. X-rays (Latarjet, 1951) and

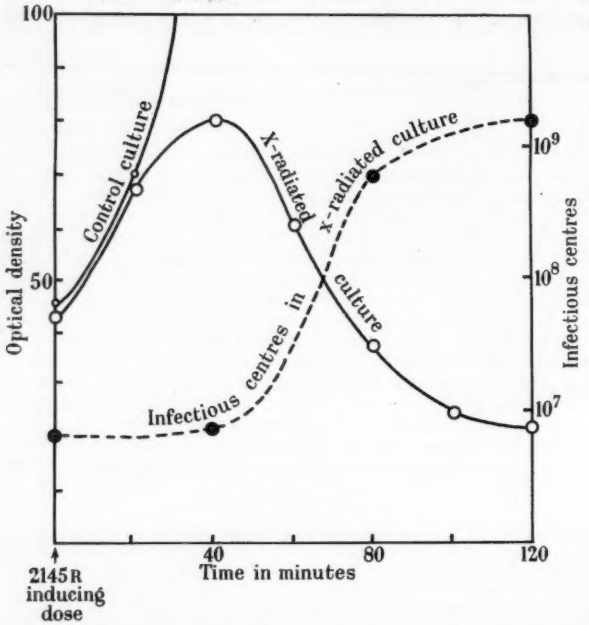


FIG. 2.—The induction of *Ps. pyocyanea* P13(8) by X-radiation. Following the inducing dose of X-rays the culture showed some residual growth before lysis took place and bacteriophage was released. 56 YC medium.

chemical mutagens (Jacob, 1952*c*; Lwoff and Jacob, 1952) have the same effect. P13(8) was shown by Jacob (personal communication) to be such a culture, being induced by ultra-violet light and by hydrogen peroxide. Induction was also obtained after X-radiation (Fig. 2).

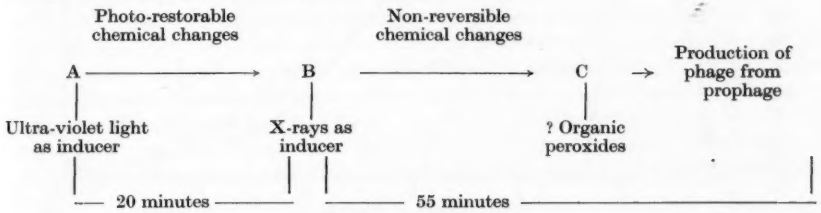
Exponential cultures containing approximately $2{\cdot}4 \times 10^8$ bacteria/ml. in broth, 56 YC and 56 lactate medium, were irradiated with doses of X-rays varying from 715 to 5720 R, diluted 1 in 3 in the same medium and their growth followed photometrically. Readings were taken every 20 min. After irradiation growth continued at almost the same rate as that of an unirradiated control for 40 min. Between this time and 60 min., depending on the dose, the growth slowed down or stopped. When the dose induced a considerable proportion of bacteria this

arrest was followed by a diminution in opacity due to their lysis. This drop continued for about 60 min. until all induced bacteria had lysed or, with the lower doses, until the growth of the remaining uninduced organisms obscured it.

The decrease in opacity was due to the lysis of the induced cells which released bacteriophage particles in large numbers into the medium.

Time of phage maturation after induction by X-rays.

Five exponential cultures of P13(8) in 56 YC were induced by four different doses of X-rays, 427, 712, 1424 (twice) and 2848 R. Samples were removed at different intervals after irradiation and titrated for infectious centres. A sudden increase in infectious centres occurred in four experiments between the 54th and 57th minute after induction and in one between the 51st and 54th minute. This is shorter than the latent period following ultra-violet irradiation (75–80 min.) but similar to that found by Jacob (personal communication) after induction by H_2O_2; *i.e.*, 60 min. He considers that ultra-violet light produces photo-restorable lesions which are responsible for induction. As he has found, induction by ultra-violet light is only reversible by ordinary light for the first 20 min. or so. It takes this time for these reactions to act upon the systems which X-rays and H_2O_2 activate at once and which perhaps lead via the intermediary of organic peroxides to the induction of prophage and the development of mature phage. This could be expressed diagrammatically as follows:



X-ray induction of P13(8) was not found to be reversed by light.

The increase in infectious centres after lysis varied from 40- to 160-fold indicating that the induced bacteria yielded on an average 40 to 160 phages each (burst size).

Induction curve.

In order to determine the optimum dose for induction exponential cultures were irradiated with different doses of X-rays and the percentage of cells induced at each dose calculated by plating for infectious centres. Before irradiation the cultures in 56 YC were centrifuged at 5000 r.p.m. for 5 min., the supernatant containing free phage removed and the surface of the deposit washed gently with 1 ml. saline and then re-suspended in 56 YC. The washed suspension was then diluted in 56 YC to give 10^5 bacteria per ml. and X-rayed. Samples were removed after different doses and plated out for infectious centres. Reversion of induction in P13(8) on plating did not occur with X-rays as it had after the ultra-violet light induction of this strain or of K12 (Weigle and Delbruck, 1951). Marcovitch (personal communication) has also found that induction of *Bact. coli* K12 by X-rays was not reversed by plating out during the latent period.

The induction curve consisted of four parts, (1) a steep upward slope due to a rapid increase in the number of cells induced; (2) a plateau containing the point of maximum induction; (3) a steep downward slope; and (4) a slope parallel with that of free phage (Fig. 3).

The curve showed that the number of cells induced rose to a maximum and then rapidly declined as the dose was increased until a level was reached at which the number of cells induced was masked by the residual phage in the preparation. The optimum dose for induction with X-rays as obtained from these curves was between 2700 and 3600 R.

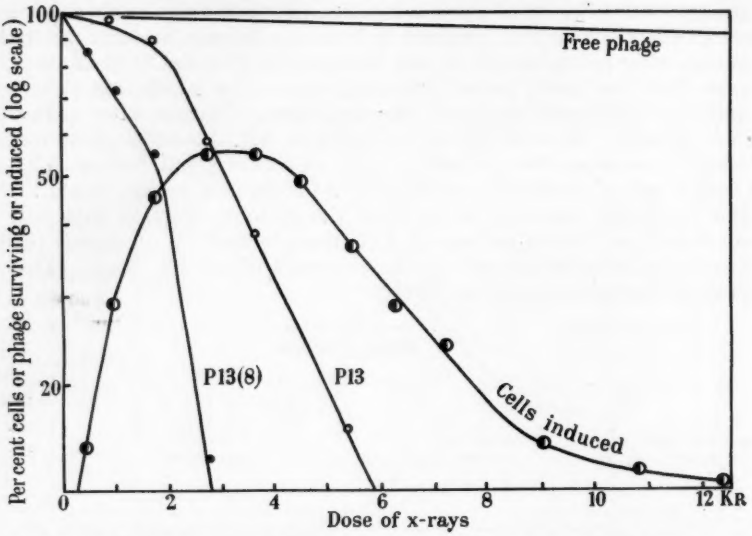


FIG. 3.—Survival and induction curves of *Ps. pyocyanea* P13(8) and the survival curve of strain P13 following low doses of X-rays. Initially, with increasing amounts of radiation more and more cells were induced to produce phage but after 50–60 per cent of the cells had been induced there was a rapid decline in the number producing phage. The full extent of this was masked by the residual phage left in the culture. 56 YC medium.

The maximum proportion of bacteria induced varied between 50 and 60 per cent in these experiments whereas ultra-violet light induced almost 100 per cent of cells.

Lysis following X-radiation of P13.

This strain has been shown by Jacob (personal communication) to lyse slowly after ultra-violet radiation but until recently no phage or antibacterial substance had been demonstrated to be released. Lysis also occurred after X-radiation but the doses needed were higher than those needed for induction and lysis of P13(8). Following irradiation with from 3600–4300 R growth continued for about 40 min., then slowed down and lysis began after about 60 min., as judged photometrically. This lysis continued somewhat more slowly than in P13(8) until a point was reached at which the turbidity of the culture remained constant

or else growth of uninduced cells masked any further decrease in opacity. Although a dose of 4000 R generally produced some lysis of P13, those of 7000–43,000 R always induced lysis either in 56 YC or, less well marked, in broth. At 86,000 R slight induction occurred but at 180,000 R lysis started immediately after irradiation and no residual growth took place.

Effect of X-radiation of P13 on phage production.

(a) *Capacity to support phage growth.*—Rouyer and Latarjet (1946) showed that with *Shigella paradysenteriae* a large dose of X-rays (400,000 R) did not stop phage production although, as judged by colony count, only 10^{-5} of the cells survived. Similar findings after ultra-violet radiation were reported by Anderson (1948) with *Bact. coli* B and phage T2, and by Benzer and Jacob (1953) with *Bact. coli* C and phage λ. X-radiation of P13 gave a different picture.

Exponential cultures of P13 in 56 YC and in broth were irradiated with increasing doses of X-rays and then infected with phage P8 at multiplicities of about 1/20 to 1/80. After allowing for adsorption to take place at 37° for 10 min. the cultures were centrifuged to remove unadsorbed phage and the deposits re-suspended in nutrient medium; their densities were checked photometrically and the number of infectious centres surviving after each dose of X-rays determined.

The power to initiate growth of phage was not affected appreciably by doses up to 3600 R but with bigger ones it declined rapidly until 21,000 R or so was reached. After that the level of residual unabsorbed phage masked any further fall. The slope of this curve was not quite so steep as that for colony production indicating the capacity to produce phage was more resistant than that of forming colonies. This difference in resistance was not so marked as it was in the case of ultra-violet radiation (Benzer and Jacob, 1953). In P13(8) the downward slope of the induction curve was less steep than that of the survival of non-induced cells (P13) indicating in this strain also that cells unable to form colonies could still produce phage.

The latent period in those cells capable of producing phage was lengthened from the normal of 65 min. to between 70 and 90 min. depending on the dose. The yield as calculated from a single growth step experiment was also reduced but owing to the level of residual unabsorbed phage this did not necessarily indicate an over all reduction in burst size.

(b) *Power to adsorb phage.*—The supernatant fluids from the previous experiments were titrated for free phage and the percentage adsorbed by the irradiated bacteria calculated. The power to adsorb phage was found to fall off very much more slowly than the power to produce phage or colonies indicating that it was but little affected by X-rays. Dead cells could adsorb phage nearly as efficiently as living ones (Fig. 4). The power to adsorb phage was lost almost at the same rate in broth as in 56 YC.

The effect of phage infection on the lysis of irradiated cells of P13.

Exponential cultures of P13 were irradiated with different doses of X-rays and the effect on the lysis of the different samples, after the addition of phage at a multiplicity of 10/1, followed photometrically. The curves of lysis were almost identical in both infected and uninfected cultures suggesting perhaps that, although adsorption occurred, development of even incomplete particles was blocked.

The effect of irradiation on lysis after infection with phage.

Cultures of P13 were infected with phage at a multiplicity of 10/1 and irradiated 25 min. after infection with doses from 7100 to 86,000 R. Lysis occurred in cells receiving up to 43,000 R at the same time as in the unirradiated control and to almost the same extent. However, in the L.L. curve recorded in the next paragraph, it was found that these doses inhibited infective phage production in an increasingly large proportion of cells. But as lysis of the bacteria was not affected, some reactions, perhaps the production of incomplete phage, must have continued and brought this about without the release of infective bacteriophage.

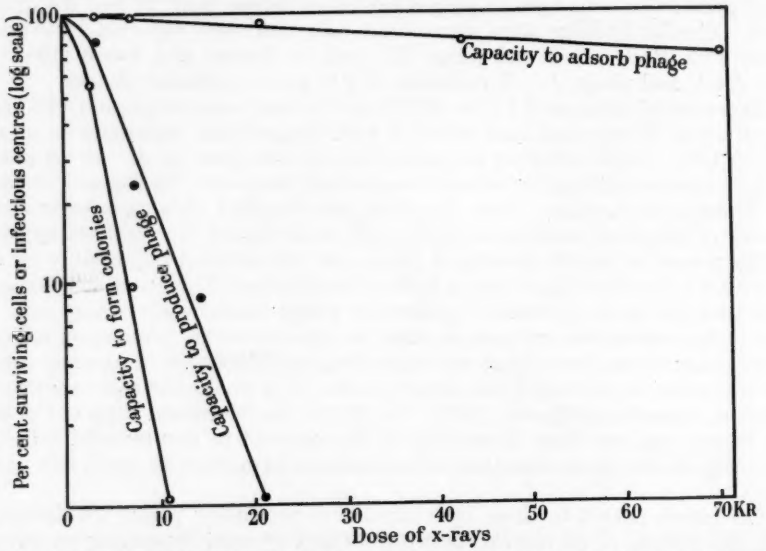


FIG. 4.—Curves of the " capacity " of P13 to adsorb phage and to produce phage and colonies after X-radiation. The cells were capable of producing phage after doses of radiation that prevented them multiplying and producing colonies. The capacity to adsorb phage only diminished slowly with increasing radiation. 56 YC medium.

Luria-Latarjet (L.L.) curves of phage 8 in P13.

The resistance of developing phage to X-rays after infection of *Ps. pyocyanea* P13 was tested by removing samples at regular intervals during the maturation period. The number of infectious centres surviving in these samples after irradiation was plotted against the dose of X-rays (Fig. 5). As no antiserum was available phage was added to the bacterial suspension (10^9 organisms per ml.) at a multiplicity of 1/10–1/20, the mixture agitated at room temperature for 10 min. and then centrifuged at 7500 r.p.m. for 5 min. in the small head of an international centrifuge. The supernatant fluid was removed, the deposit re-suspended in 56 YC, diluted to give approximately 2×10^4 bacteria/ml. and incubated at 37°. Samples were removed at 30, 40, 50, 60 and 70 min. after infection, irradiated with doses of from 1000–250,000 R and titrated for survival of infectious centres. The latent period was increased from the normal 65 min. to 80 min.

by this procedure indicating that 30 min. at room temperature was equivalent to 15 min. at 37°. In the figure the times have been calculated as though the whole experiment had been done at 37°. The curves of resistance were found to be somewhat similar to those obtained with ultra-violet light by Benzer and Jacob (1953). After a dose of 3000 R there was a rapid decrease in resistance to X-rays, the number of infectious centres falling off until the base line of residual phage was

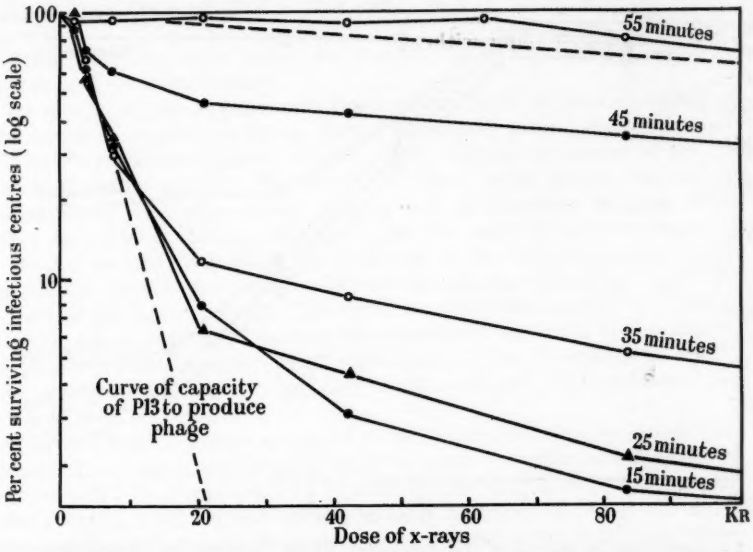


FIG. 5.—Luria-Latarjet curves of *Ps. pyocyanea* P13 obtained by X-radiation. These rays interfered with the production of mature phage during the first half of the cycle; but with the appearance of the latter in the cells the effect on it became obscured as the mature particles accumulated in the cells. This was indicated by the rising of the last part of the curve during the second half of the maturation period. 56 YC medium.

reached. This level remained constant for the first half of the latent period but at 35 min. a small rise had occurred indicating an increase in the number of resistant intracellular particles, presumably the beginning of the formation of mature phage in the bacteria. This level had risen still further and a plateau had appeared between 45 and 55 min. when the last reading was taken, indicating the appearance of multiple phage particles in the bacterial cell.

Evidently the factor in the cell which inhibited phage production in its vegetative form was effective throughout the cycle until mature phage was present in the cell. These particles appeared first at the beginning of the second half of the latent period and had the same resistance to X-rays as free phage.

Luria-Latarjet curves of P13(8) following induction.

After induction of P13(8) by a dose of 3400 R, cultures were incubated at 37° and removed at different times and irradiated as in the case of infected P13. From 5 to 30 min. the decrease in resistance was the same until the curve flattened

out at a level considered to indicate residual free phage left in the preparation after centrifugation. This last part of the curve rose slowly from 35 to 45 min. by which time a plateau had appeared indicating that the majority of cells contained multiple phages at that time (Fig. 6).

As with P13 the same factor inhibiting phage production in the cells was found to be active until insusceptible mature phage had been formed.

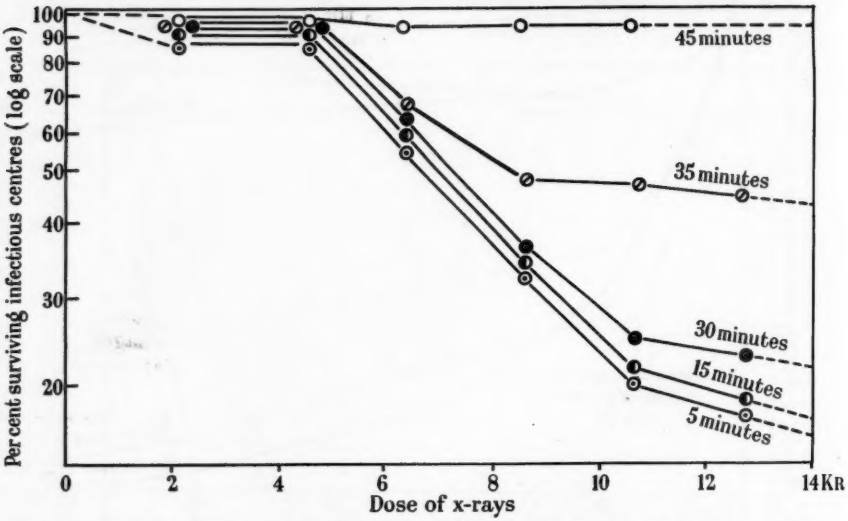


FIG. 6.—Luria-Latarjet curves of *Ps. pyocyanea* P13(8) obtained by X-radiation. The resistance of induced developing phage followed the same pattern as phage in *Ps. pyocyanea* P13 except that mature phage appeared somewhat earlier. The level of residual phage appeared to be higher in this system because of the lower proportion of cells producing phage after induction of P13(8) than after infection of P13. 56 YC medium.

DISCUSSION.

This study was undertaken as another contribution of radiobiological methods to the problem of virus production and to supplement some of the experiments of Jacob (1952*a*, *b*) and Benzer and Jacob (1953), on the effect of ultra-violet radiation on the same two strains of *Ps. pyocyanea*. Their results were in general paralleled by those found with X-radiation. The temperate phage P8 was found to be more resistant to X-rays than virulent phages of the same size and its induction in its lysogenic host was produced by X-rays in different media. However, certain differences between the effect of the two types of radiation were noted. The latent period following induction by X-rays was considerably shorter than that by ultra-violet light. On the basis of Jacob's hypothesis this has been explained by suggesting that X-rays act at a point in the induction mechanism which is only reached after 20 minutes of synthesis of the photo-restorable mechanism induced by ultra-violet light.

X-ray induction of P13(8) was not affected subsequently by light nor was it

reversed by plating out during the latent period while ultra-violet light induction is counteracted by both. This difference has already been noted by Marcovitch (personal communication) in *Bact. coli* K12. However, X-rays were not capable of producing active phage in as large a proportion of cells as ultra-violet light (95 to 100 per cent).

X-ray induction of P13(8) was limited by the " capacity " of the cells to produce phage, this causing the rapid decrease in phage-producing cells after the maximum had been reached. This slope of the curve was the same as the " capacity " of P13 to produce phage and as the steep part of the L.L. curves suggesting that the same factor was interfering with phage production in both lysogenic and sensitive strains.

The results of the experiments done in the hope of demonstrating variation in resistance of vegetative phage during the latent period were obscured by the factor in the bacterium-phage complex which limited the power of the bacterium to produce active phage. The results obtained with X-rays were of the same order as those with ultra-violet light in P13 and similar to those with P13(8) (Benzer and Jacob, 1953). With X-rays the survival of vegetative phage in P13 always seemed to parallel the capacity of the cells to produce phage while on the other hand it was more sensitive than this to ultra-violet light during the first part of the latent period. However, no conclusion could be drawn as to any change in phage resistance as was demonstrated by Luria and Latarjet (1947), Latarjet (1948) and Benzer (1952) with some of the T bacteriophages. Mature phage, as one would expect, had the same resistance to X-radiation whether it was intra- or extra-cellular.

In P13(8) mature phage appeared to be formed 20 minutes before lysis began, while in P13 it appeared from 20 to 30 minutes in different experiments. This variation could be explained by the condition of the experiments involving P13. It was necessary during adsorption to keep the bacteria at room temperature, as placing them in an ice-bath destroyed a large number of the bacteria and inhibited phage production. All phages would not be adsorbed or start developing at exactly the same time and so the time of appearance of mature phage would not be so sharply defined as in P13(8). On the other hand all cells would be induced at about the same time. This would explain the apparent difference in the time of formation of mature phage before lysis in the two systems and would suggest that it was about the same in both. Should this be so it would indicate that the production of phage from prophage takes less time than the process " adsorbed phage to phage."

In infected P13 irradiation after 25 minutes did not stop lysis of the bacteria after the usual latent period although very little free phage was released by the irradiated cells. Perhaps the factor which prevented the development of active phage interfered with the desoxyribonucleic acid (DNA) and not with protein metabolism. That some such reaction might take place is suggested by the effects of proflavine on phage production; Foster (1948) has indicated that this drug blocks a late reaction necessary for the production of active phage although the bacteria lyse at the normal time. Also De Mars, Luria, Fisher and Levinthal (1953) have shown that proflavine, although inhibiting infective phage, does allow the release of protein particles (or " doughnuts ") devoid of DNA and very similar to phage ghosts. On the other hand phage P8 adsorbed on irradiated cells of P13 does not increase the usual lysis of these cells, suggesting that the

factor inhibiting phage production can block both phage DNA and protein synthesis if it can be activated before phage development has started.

Thus the effects of ultra-violet light and X-rays have once again been shown to be similar in many respects although X-rays may sometimes produce the same end-result by exciting systems not directly susceptible to the action of ultra-violet light.

SUMMARY.

The sensitivity to X-rays of a temperate phage and its two host strains of *Ps. pyocyanea*, one sensitive and the other lysogenic, has been investigated. The effect on induction of the lysogenic strain, the behaviour of the phage in the X-radiated sensitive strain and the L.L. curves of phage development in both have been studied and similarities between them and those of ultra-violet light observed.

My thanks are due to Dr. A. Lacassagne, Director of the Institut du Radium, Paris; to Dr. R. Latarjet, in whose laboratory the work was initiated, and to Dr. A. Lwoff for their very helpful advice and criticism and for suggesting the problem; and to Mr. P. Morenne for operating the X-ray machines.

My thanks are also due to Dr. W. M. Dale for permission to use the cobalt source at the Holt Radium Institute, Manchester, for some of these experiments.

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A NOTE ON THE ANTIGENIC PROPERTIES OF CORNEAL EXTRACTS.

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THE antigenic properties of the components of connective tissues have been investigated in very few instances and results have been mainly negative; thus Wormall (1944) suggested that collagen and its derivatives are not antigenic, and Humphrey (1943) was unable to prepare antibodies to hyaluronic acid even after it had been covalently bound to a protein; Glynn and Holborow (1952), however, have reported that chondroitin sulphate linked to β-haemolytic streptococci is antigenic. The activity of the constituents other than collagen and mucopolysaccharides has received very little attention. Indeed, with regard to the cornea the only report is that of Romer and Gebb (1912) who obtained rather inconsistent anaphylactic symptoms after injection of extracts of ox cornea into rabbits.

With the progress in the chemical study of connective tissue it becomes of interest to establish the identity or otherwise of the separate components of the respective tissues and the immunological test is the most sensitive means of establishing such identity. Using this method we have made a preliminary study of the antigenic capacity of corneal extracts which has been facilitated by recent information on the chemical composition of the cornea described by Woodin (1952, 1953).

MATERIAL AND METHODS.

Preparation of materials.

The aqueous extract was obtained by extracting sections of bovine cornea with distilled water at 4°; the residue from the aqueous extraction was used for the preparation of mucoids and mucopolysaccharide following the procedure described by Woodin (1952).

Bovine serum was provided by Wellcome Research Laboratories and bovine serum albumin by Armour Laboratories.

Fractionation of the aqueous extract.

The fractionation was carried out by the addition of Na_2SO_4 at 37° and was based on the scheme used by Majoor (1947) to fractionate the proteins of serum. The ppt. was collected on Whatman No. 50 filter paper and re-dissolved by dialysis. The ppt. from the 18·5 per cent Na_2SO_4 solution was denatured if allowed to dry on the filter paper and hence it was kept covered by a little solution. Consequently this ppt. was slightly contaminated by the later fractions.

Analysis of the corneal extracts.

The analyses were carried out on portions of the dialysed solutions of the corneal material. The solution of the 18·5 per cent Na_2SO_4 ppt. from the aqueous extract was not stable and some of it precipitated on dialysis. The dialysed suspension was dried and the analysis

carried out on the solid material; no correction was made for that inorganic material not removed by dialysis, but tests were applied to ensure freedom from SO_4. Nitrogen was determined by Kjeldahl digestion using a selenium catalyst followed by distillation in a Markham still. Hexosamine was determined by the method of Elson and Morgan (1933), after hydrolysis in 5 N HCl for 6 hr. (Woodin, 1952). Ester sulphate was determined gravimetrically by precipitation with $BaCl_2$ after hydrolysis in 5 N HCl for 24 hr.

Immunisation procedure.

Groups of 3 rabbits were immunised by intravenous injection of the aqueous extract. The extract was a poor antigen and it was necessary to give a weekly injection of 2 ml. of a 1 per cent solution for 12 weeks to obtain sera of sufficient activity. The dose of extract could not be increased because it was found that, as detectable antibody appeared, the animals became liable to anaphylactic shock if more than 25 mg. of antigen were injected.

Tests for antibody.

Precipitin tests were used for the detection of antibody. The tests were carried out at room temp., 37° and 56°, readings being made after 6 and 24 hr., max. titres being found at 37°. In all experiments a chessboard titration was made to determine the optimum antigen concn. as well as the serum titre.

Analysis of supernatants after absorption of the antisera with serum proteins and corneal fractions was found to be difficult, both because the antiserum was of low titre and because fractionation of the aqueous extract by chemical means did not lead to a sharp separation of the antigens. To confirm the tube precipitation tests, therefore, the agar diffusion methods described by Oudin (1948) and Oakley and Fulthorpe (1953) were used.

RESULTS.

Composition of the corneal extracts.

The aqueous extract of cornea contains several substances. On electrophoresis at pH 7, four components can be distinguished of which the fastest contains the acid mucopolysaccharide. It has been shown that a similar phenol-insoluble mucoid is obtained from the water-extracted sections as from the whole cornea, which suggests that the mucopolysaccharide is in the same state of combination in the aqueous extract as in the soluble mucoid.

On addition of Na_2SO_4 to the aqueous extract a ppt. is obtained in the Na_2SO_4 concn. range 0–27 per cent; little further ppt. is obtained on increasing the salt concn. For convenience the fractions collected were those produced in the Na_2SO_4 concn. 0–18·5 per cent, 18·5–27 per cent and that remaining soluble in saturated Na_2SO_4; these fractions have been called I, II and III respectively.

TABLE I.—*Composition of the Extracts of Ox Cornea used in the Tests for Presence of Antibody.*

Material.	Nitrogen per cent.	Hexosamine per cent.	Ester sulphate per cent.
Aqueous extract of cornea	13·1	7·3	0·53
0–18·5 per cent Na_2SO_4 ppt. of aqueous extract. (Fraction I)	14·0	3·0	0·2
18·5–27 per cent Na_2SO_4 ppt. of aqueous extract. (Fraction II)	12·3	9·8	0·7
Fraction of aqueous extract soluble in sat. Na_2SO_4. (Fraction III)	4·0	3·4	0·7
Phenol-insoluble mucoid	8·7	18·4	3·8
Soluble mucoid	11·2	12·7	2·1

Table I shows the composition of the fractions obtained in this way, and the composition of the mucoids and the aqueous extract.

Antigenic activity of the aqueous extract.

The preliminary chessboard experiments to determine the optimal concn. of antigen to be used showed that the highest titres were obtained with an antigen concn. of between 0·05 and 0·1 per cent. After 12 weekly injections of 20 mg. of aqueous extract 6 rabbits' sera gave titres of 160.

Precipitin tests were performed using the 18·5 per cent Na_2SO_4 and 18·5–27 per cent Na_2SO_4 fractions of the aqueous extract as antigens; these fractions reacted with the immune serum prepared against the aqueous extract to the same titre as did the unfractionated extract.

Antigenic activity of the mucoids and the mucopolysaccharide.

The serum prepared against the aqueous extract was tested for the presence of antibodies to the corneal mucopolysaccharide and the mucoids. The results are given in Table II from which it is apparent that only the soluble mucoid reacted to any extent and even then the titre was very low. To confirm that the acid mucopolysaccharide was not participating in the reaction with the aqueous extract, samples of the ppt. produced from the aqueous extract and the immune serum were collected and tested for ester sulphate with negative result.

TABLE II.—*Antigenic Activity of the Mucoids and Mucopolysaccharide assessed by the Precipitin Test.*

Material.	Range of concentrations of antigen mg. per cent.	Titre of serum.
Mucopolysaccharide	0·6 –0·0025	*Nil*
Phenol-insoluble mucoid	0·05–0·003	2
Soluble mucoid	0·05–0·003	20

Reaction with bovine serum and bovine serum albumin.

In an attempt to determine the origin of the antigenic substances in the aqueous extract of cornea the ability of the immune serum to react with bovine serum was assessed. A 1/5 dilution of immune serum reacted with bovine serum to a titre of 30,000 and a dilution of 1/25 to a titre of 6,000. Only part of the antibody could be absorbed from the immune serum by bovine serum. After three absorptions the immune serum no longer gave a ppt. with bovine serum but reacted with the aqueous extract.

Although the immune serum reacted with bovine serum, no precipitate was formed when it was tested against bovine serum albumin. In gel precipitin reactions, however, a single band of precipitation occurred, but took 72 hr. to appear.

Results of diffusion analysis.

The aqueous extract gives rise to two well defined bands with its own antiserum agar gel. By using the relationship between rate of diffusion and time (Munoz and Becker, 1950) it can be shown that these bands are due to two antigens. The formation of these two bands cannot be inhibited by previous absorption of the immune serum with bovine serum, although bovine serum itself reacts with the anticorneal serum to form four diffuse bands of precipitation.

The mucoid fractions reacted as was expected from the first precipitation tests ; thus there was no reaction between the phenol-insoluble fraction and the antisera, whilst the soluble mucoid reacted to give two bands.

The sodium sulphate fractions were next investigated and it was found that fractions I and II gave two bands, while fraction III gave only a single band.

DISCUSSION.

It is clear that in the aqueous extract of ox cornea substances are present which, when injected into rabbits, induce the formation of precipitating antibodies, and it should be possible by using similar immunological methods to compare the water-soluble constituents of the cornea with those of other connective tissues. The main limitation of technique is that the production of sera of high titre is made impracticable by the tendency of the rabbits to anaphylactic shock following the injection of the corneal extracts. It is possible that more active antisera could have been produced by subcutaneous immunisation particularly if adjuvants had been added. Such methods cause local inflammation or even necrosis, and when dealing with connective tissue antigens it was thought best to avoid these reactions ; hence the intravenous route was chosen.

The nature of the corneal antigens is not yet completely clear but there seems no doubt that there are at least two groups ; one is formed by serum proteins and the other by components of the cornea itself. The corneal mucopolysaccharide is not antigenic and in this property it resembles hyaluronic acid. Attempts to follow the antigens by fractionation of the aqueous extract with sodium sulphate have not been satisfactory because chemical fractionation is insufficiently clear-cut to separate fractions I and II immunologically. Fraction III did not react in the fluid precipitation test but formed a single band with the diffusion method and probably contains serum albumin.

SUMMARY.

The water-soluble components of ox cornea induce the formation of precipitating antibodies in rabbits.

The corneal mucoids and mucopolysaccharide do not react with immune serum.

Some of the antigenic constituents of ox cornea are also present in ox serum.

Two antigens from the corneal extract appear to be derived from the connective tissue constituents of the cornea.

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SYNERGISM BETWEEN STREPTOMYCIN AND PENICILLIN.

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THE phenomena of synergism and antagonism between pairs of antibiotics are well established from *in vitro* and animal-infection experiments (Robbins and Tompsett, 1949; Jawetz, Gunnison and Coleman, 1950). As yet there is no satisfactory explanation of either phenomenon, although several hypotheses have been presented. For example, it has been suggested that bacteriostatic agents, *e.g.*, chloramphenicol, are antagonistic in combination with penicillin because they stop growth, (Editorial, 1950) and penicillin acts best on growing organisms (Hobby and Dawson, 1944). Miles, Coleman, Gunnison and Jawetz (1951) discussed several hypotheses which have been advanced to explain synergism but considered that only two of these were in agreement with the known facts. These are (*a*) that one drug may increase the permeability of the cell wall to the other, or (*b*) potentiation may occur through the simultaneous blocking of alternative enzymic pathways. Studies in this laboratory (Rowley, Cooper, Roberts and Lester Smith, 1950) using ^{35}S-labelled penicillin, have shown that penicillin-sensitive organisms take up more penicillin than do penicillin-resistant ones and the degree of sensitivity and penicillin uptake are roughly proportional. Using labelled penicillin and minimal streptomycin concentrations, it should be possible to demonstrate the existence of a mechanism such as (*a*) above, provided that synergism in this case was the result of an increase in penicillin uptake perhaps due to some action of the streptomycin on the bacterial surfaces.

This paper describes experiments designed to test this hypothesis. The uptake of radioactive penicillin by two strains of *Streptococcus faecalis* has been measured in the presence and absence of synergistic streptomycin concentrations. The results indicate quite clearly that the uptake of penicillin by these organisms was not altered by streptomycin over the range of concentrations at which synergism was observed to be most marked.

MATERIALS AND METHODS.

Two strains of *Str. faecalis* (7 and 8), isolated from cases of bacterial endocarditis, were used in these studies. Both strains were unaffected by 1 unit of penicillin/ml. but were slowly killed by 4u./ml., while addition of a sub-inhibitory (25 μg./ml.) amount of streptomycin to the 4u./ml. penicillin greatly increased the rate of killing.

The organisms grew well in the broth used, which contained in 1 l., 1 g. Lab. Lemco, 2 g. "Yeastrel" (Brewers Food Supply Co., Ltd., Edinburgh), 5 g. peptone and 5 g. NaCl. The pH was adjusted to 7, and the broth was autoclaved at 15 lb. for 20 min.; 5 g. glucose in 20 ml. water was autoclaved separately and added with 100 ml. horse serum to each litre of the broth. The cells were harvested by centrifuging after 14–16 hr. growth at 37°, and

* Working with a personal grant from the Medical Research Council.

were washed twice with distilled water in the experiments with resting cells. For experiments with growing organisms the cells were re-suspended in fresh broth at pH 7 containing 0·04 M phosphate buffer.

The radioactive penicillin was prepared bio-synthetically as described by Rowley *et al.* (1950). That the radioactive and biological activities resided in the same fraction was shown by counter-current distribution analysis (Cooper, Clowes and Rowley, unpublished results). We are indebted to Dr. E. Lester Smith for a generous supply of crystalline radioactive penicillin which enabled us to conduct experiments on growing cells.

Bacterial concentrations were determined turbidimetrically using a " Spekker " absorptiometer calibrated in terms of bacterial dry wt. for that particular strain.

Measurement of penicillin uptake by resting cells.

Two series of tubes were prepared, one containing the required dilutions of radioactive penicillin in 0·04 M phosphate buffer adjusted to pH 6·8 and the other containing the same dilutions of penicillin plus the required dilutions of streptomycin. To each of these tubes was added a sample of a washed suspension of *Str. faecalis* to give a final concentration of 2–4 mg. bacterial dry wt./ml. These suspensions were kept at room temperature for 30 min., centrifuged and the deposit was washed by suspending in distilled water and re-centrifuging four times. The organisms were then made up to the original volume with distilled water and 0·2 ml. samples from each tube were transferred to duplicate planchettes and allowed to dry for radioactive assay. Further samples taken for turbidity measurements showed there was no significant loss of organisms during the manipulations. A sample of the final wash water was assayed for radioactivity to ensure that washing had been completed.

To study the effect of penicillin and streptomycin alone and in combination on the viability of a " resting " culture of *Str. faecalis* (a 24-hr. broth culture), a series of tubes was prepared containing 9 ml. of this broth culture and 1 ml. of streptomycin and/or penicillin solution. The tubes were re-incubated and samples were removed at intervals for viable counting by the method of Miles and Misra (1938). The dilutions for each count were allowed to drop on four separate blood agar plates and were incubated for three days before counting.

Measurement of penicillin uptake by growing cells.

Two series of dilutions, one of radioactive penicillin and the other of radiopenicillin plus streptomycin, were prepared in the enriched medium used for growing the *Str. faecalis*. These tubes were inoculated with bacterial suspensions to give a final concentration of 0·2 mg. bacterial dry wt./ml. This was chosen as the greatest concentration which would allow further growth of the organisms. All the tubes (which had previously been kept at 37°) were incubated at 37° for periods of 1–8 hr. During this incubation samples of each suspension were removed for assay of radioactive uptake, turbidity and viable counts as previously described, the final suspension for radioactive assay being adjusted to 2 mg. bacterial dry wt./ml.

RESULTS.

Resting cells.

" Resting " cell suspensions were used as in previous work on uptake of penicillin by staphylococci (Rowley *et. al.*, 1950). Fig. 1 shows that presence or absence of streptomycin makes no difference to the rate or final level of penicillin uptake by resting cells, and Table I that rate of uptake is not altered. This is perhaps to be expected, as when viable counts were made there was no indication of synergism occurring in these resting suspensions. In one particular experiment resting suspensions of *Str. faecalis* 8 were incubated for 7 hr. in concentrations of 0, 4, 8 units penicillin/ml. and using 25 μg. streptomycin/ml. without change in the viable count. Uptake studies were therefore made on growing cells under conditions where the maximum amount of synergism between the two drugs could be simultaneously observed.

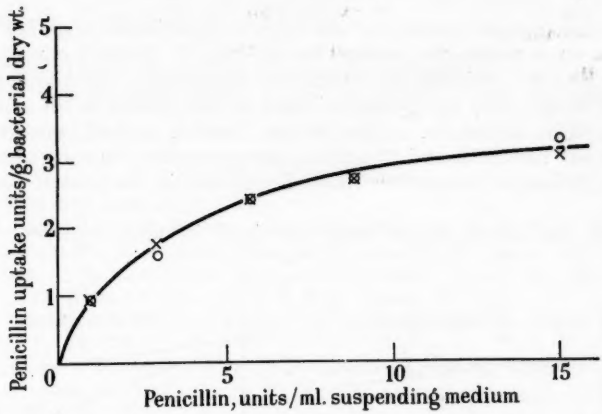

FIG. 1.—Radiopenicillin uptake by resting *Str. faecalis* cells (Strain 7). o–o, penicillin alone, x–x penicillin plus 25 μg./ml. streptomycin (second expt. of Table I).

TABLE.—*Uptake of Penicillin by* Str. faecalis *in Presence or Absence of Streptomycin.*

Strain No.	State of organisms.	Hours' contact.	Streptomycin μg./ml.	Penicillin units/ml. 1.	2.	3.	4.	6.	8.	9.	10.	15.
				Penicillin uptake units/g. bacterial dry wt.								
7	Resting cells	½	0	0·33	—	1·4	—	2·0	—	—	2·44	—
			25	0·8	—	1·46	—	2·0	—	—	2·59	—
7	,,	½	0	0·96	—	1·65	—	2·5	—	2·81	—	3·4
			25	1·0	—	1·82	—	2·42	—	2·83	—	3·17
8	,,	5 min.	0	—	0·76	—	—	—	—	—	1·68	—
			25	—	0·74	—	—	—	—	—	1·74	—
		1 hour	0	—	1·31	—	—	—	—	—	2·58	—
			25	—	1·32	—	—	—	—	—	2·5	—
8	,,	¾	0	—	1·22	—	1·33	—	—	—	1·66	—
			50	—	1·14	—	1·48	—	—	—	2·4	—
			100	—	1·1	—	1·5	—	—	—	2·4	—
			600	—	1·1	—	1·5	—	—	—	2·5	—
8	,,	¾	0	0·81	1·23	—	1·87	—	2·41	—	—	—
			100	0·86	1·23	—	1·81	—	2·46	—	—	—
8	,,	1¼	0	—	1·72	—	2·30	—	3·00	—	—	—
			100	—	1·66	—	2·24	—	3·21	—	—	—
8	Growing cells	1¼	0	1·35	—	—	2·26	—	—	—	—	—
			25	1·28	—	—	2·70	—	—	—	—	—
		3	0	2·10	—	—	4·36	—	—	—	—	—
			25	1·71	—	—	2·24	—	—	—	—	—
8	,,	½	0	1·43	—	—	2·23	2·81	—	—	—	—
			25	1·48	—	—	2·59	2·63	—	—	—	—
		1¼	0	1·65	—	—	2·91	3·84	—	—	—	—
			25	1·72	—	—	2·61	3·15	—	—	—	—

Growing cells.

The penicillin uptake curves for one typical experiment, together with viable counts on the same suspension, are plotted in Fig. 2. From a comparison of the viable counts at zero penicillin concentrations in presence and absence of streptomycin, it can be seen that streptomycin alone allows growth to the same extent as the control with no antibiotic. After 80 min. there is marked synergism between 4 and 6 units/ml. penicillin and 25 μg./ml. streptomycin ; in spite of this, there is no significant difference between uptakes of penicillin in the presence or absence of streptomycin.

Essentially the same results were obtained in four separate experiments (see Table).

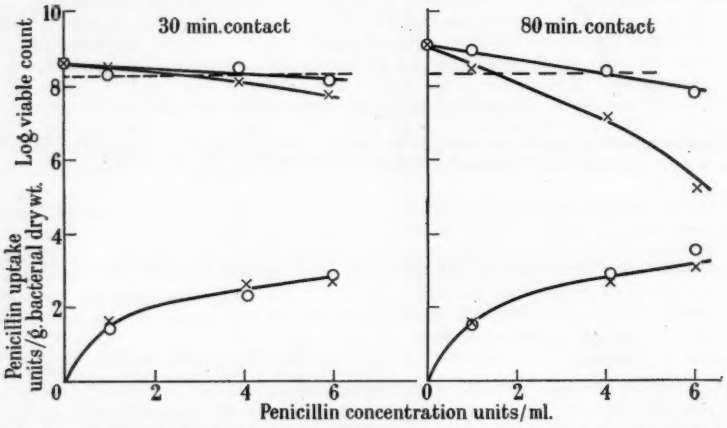

FIG. 2.—Radiopenicillin uptake and viability of growing *Str. faecalis* (Strain 8) with and without 25 μg./ml. streptomycin. o–o, penicillin alone, x–x, penicillin plus streptomycin. - - -, viable count at zero time (last expt. of the Table).

DISCUSSION.

Of the two alternative mechanisms proposed by Jawetz and his colleagues (Miles *et al.*, 1951) to account for synergism between antibiotics, we have concerned ourselves solely with the suggestion that streptomycin may potentiate penicillin by increasing the permeability of the organism to penicillin. In this case the lethal action would be due to penicillin alone and would be effected by producing a higher concentration of penicillin at its site of action. Our present experiments show that one cannot account for the synergism between streptomycin and penicillin on the basis of the effect being due to an increase in penicillin concentration at its site of action.

SUMMARY.

One of the hypotheses put forward to account for antibiotic synergism is that one antibiotic may potentiate the action of another by increasing its effective concentration at the site of action. In the case of the synergistic pair penicillin

and streptomycin, the present work shows that streptomycin does not increase the concentration of penicillin on the bacteria.

We are grateful to Sir Alexander Fleming for his continued encouragement, and to Professor R. Cruickshank and Dr. P. D. Cooper for helpful criticism and advice.

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BROMSULPHTHALEIN RETENTION IN YOUNG RATS ON DIETS PRODUCING LIVER NECROSIS

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AT a time when we were trying to produce pathological changes in the livers of rats with the object of testing the protective effect of tocopherols (Selzer, Parker, McKenzie and Linder, 1951), tests of liver function were made on the blood obtained when the animals were killed. These tests comprised a bromsulphthalein test, the thymol turbidity and flocculation, the colloidal gold and sometimes the albumin and globulin fractions. It was found that even in animals in which the diet failed to cause liver lesions after hundreds of days there was a quite definite and regularly occurring failure to eliminate bromsulphthalein. In this paper these results are examined and another experiment is reported to show how soon this abnormality appears.

MATERIALS AND METHODS.

Experiment 1.—Albino rats which had been reared as described in the above paper and weighing 30–66 g. were used. The diet was one of mealie meal and sour milk described by Gillman, Gillman, Mandelstam and Gilbert (1945) which in our hands did not regularly give rise to hepatic lesions. In fact many of the rats survived for over 200 days and no significant lesions were found when they were killed. Tests were made on 25 rats killed after 180–340 days on diet and on a group of eight which were bled from the tail after 100–130 days on diet. Most of these survived for another year.

Experiment 2.—The diet used was one of corn starch and food yeast suggested by Gillman (personal communication, 1949) which was described in our previous paper and found to cause liver lesions with great regularity when it was given to rats weighing 30–66 g. If the rats were larger at the beginning of the experiment many escaped. For the present purpose it was decided to use larger rats : these weighed from 69–153 g.

Bromsulphthalein.—The bromsulphthalein test was adapted from that used by Casals and Olitsky (1946) on mice. Bromsulphthalein (BRS), as supplied in 50 mg./ml. strength, was diluted 1/10 with sterile normal saline and a dose of 1 ml./100 g. rat was injected at body temperature into the peritoneum ; the dose was thus 0·05 mg./g. Thirty minutes later the rats were killed by cutting the carotid arteries after stunning. The blood from each was collected through a glass funnel into a centrifuge tube and allowed to clot. The BRS concentration was measured in the serum and the serum left over was used for other liver function tests.

Saline (1·8 ml.) was placed in a small absorption tube for use with a Klett-Summerson photoelectric absorptiometer, and 0·2 ml. of serum was added. The solution was acidified with 0·05 ml. 10 per cent (v/v) HCl and after 5 min. the extinction was read using the 56 filter (reading A). The maximum transmission of this filter was measured on a Unicam spectro-photometer and found to be at a wave length of 556 mμ. To develop the colour 0·05 ml. of 10 per cent NaOH was added and a second reading was taken with the same filter (reading B). The extinction of the diluted serum containing no BRS was increased

* In receipt of a grant from the South African Council for Scientific and Industrial Research.

1·15 times by alkalinisation ; reading A, which was very small unless there was haemolysis, was multiplied by this factor and subtracted from reading B. The extinction of a 1 mg./100 ml. solution of BRS was 0·70 and it was found to obey Beer's law over the range required (0·1 to 1·0 mg. per cent). The serum was diluted 10·5 times so the serum BRS was $\frac{B - 1{\cdot}15\,A}{0{\cdot}70} \times 10{\cdot}5$ mg./100 ml.

Maclagan's methods (1944, 1946) were used for the thymol and gold tests, and the serum proteins were determined by the method of Wolfson, Cohn, Calvary and Ichiba (1948).

RESULTS.

Necrosis.—In Exp. 1 we found a single microscopic area of necrosis in each of three rats and in one of these there was a definite fibrosis ; this failure to produce massive liver necrosis or fibrosis with this diet was in accord with our experience. In Exp. 2 necrosis began to appear after 40 days and in rats dying or killed after that time massive necrosis or post-necrotic scarring was found in 25 out of 45. No rat died of necrosis before the 52nd day on diet. The incidence of necrosis was 100 per cent in other experiments with this diet, but in this experiment the animals were larger, and larger animals are known to be more resistant.

Bilirubin.—No quantitative tests were made for bilirubin but the sera were pale and in no case was there an obvious increase in bilirubin.

Thymol and gold tests.—These tests were performed on 54 rats. In one the thymol turbidity was 2·5, in two others 2, in four more 1·5 and in all the rest less than 1. Flocculation did not occur. The colloidal gold changed to 1 in six sera but with the rest there was no change at all. The iodine slide test (Mallen, 1950 ; Woolf, 1951) was applied to 34 sera and was negative in all.

Serum proteins.—The normal concentration of serum proteins in rats on our standard animal house diet was : albumin 3·77 per cent (S.D. 0·42), globulin 2·47 per cent (S.D. 0·23). The serum proteins were estimated in 14 rats in Exp. 1. In the three rats which survived for over 330 days the globulin was increased to 3·7, 3·0 and 3·9 per cent and the albumin was only 2·7, 3·1 and 2·0 per cent. In none of the others was the globulin above 2·7 per cent or the albumin below 3·0 per cent. In only two of the rats in the 250–320 day group was this determination made.

Bromsulphthalein.—This test was performed as described on 33 normal rats of weights similar to those used in the experiments. The mean bromsulphthalein (BRS) content of the serum after 30 min. was 1·07 mg. per cent with a standard deviation of 0·43. No variation of the BRS level with body weight was detected. It was assumed that the BRS level of a normal rat would be very unlikely to exceed 2·36 mg. per cent., *i.e.*, the mean plus three times the standard deviation.

In the table is given a summary of the experiments which shows the mean level of BRS found after different periods on diet and its standard deviation. It also shows how many of the rats in each group retained a normal capacity to eliminate the dye.

Exp. 1 shows that on a deficient diet which seldom produced necrosis there was definite retention of BRS in 60 per cent of the animals. If the last very small group killed after 340 days is set aside, there is no evidence that the proportion of rats retaining a normal power of eliminating BRS was diminished as the time on diet increased.

In Exp. 2 the diet was much more effective in producing necrosis and a much

TABLE.—*Results of Bromsulphthalein Tests in Rats.*

Exp.	Days on diet.	Number of rats.	Serum bromsulphthalein. Mean (mg. per cent).	Standard deviation (mg. per cent).	Number within normal limits.	Necrosis or fibrosis.
1	100–130	8	4·57	4·2	2	0
	180	7	2·54	2·0	2	0
	190–250	7	3·3	2·4	2	1*
	250–320	8	2·73	1·5	3	2*
	340	3	6·7	5·7	0	0
2	10	6	5·2	3·4	1	0
	17	6	2·8	3·5	4	0
	25	6	6·05	3·1	0	0
	32	6	4·9	2·3	0	0
	38	6	6·35	3·2	0	0
	45	6	10·7	6·1	0	3
	52	6	6·7	3·9	0	1
	59	6	6·5	5·9	1	3
	Died 40 –	8	—	—	—	0
	,, 40 +	12	—	—	—	8
	Killed 60	15	—	—	—	10

* Minimal lesions.

larger proportion of the rats showed retention of BRS, and in general the degree of retention was greater. The deficient elimination appeared soon after the dieting was begun : five out of six in the group killed after 10 days showed this but in the next group, killed after 17 days, only two out of six were abnormal. After 25 days or more on diet only one rat out of 36 was able to eliminate the BRS normally.

DISCUSSION.

The removal of BRS from the blood is extensively used in the clinical assessment of liver function and experience has shown that reliance can be placed on it. Barker, Capps and Allen (1945) described it as " our most reliable test " in acute hepatitis without jaundice and as a " most valuable " test in chronic hepatitis ; they found it superior to the Hanger test, increase of serum globulin or of phosphatase. Hoaglund and Shank (1946) found it to be " particularly useful in indicating latent hepatic insufficiency " and incomplete recovery from hepatitis.

The physiological basis of the test is not so satisfactory for Cohn, Levine and Streicher (1947) found that in animals deprived of liver, kidney and intestine BRS disappeared from the blood at a quarter of the rate that it did in the intact animal. If a constant infusion of the dye was given to such eviscerated animals the BRS level in the plasma became stabilised, and therefore under such conditions the amount of dye taken up by the tissues was equal to the amount infused ; the fate of this dye is not known. By duodenal intubation in men Cantarow and Wirts (1941) recovered 67–100 per cent of the dye in the two hours following the injection, but in hepatic and biliary tract disease only 30 to 75 per cent was recovered, and this in the absence of abnormal retention in the blood. Storing of the dye in the liver and possibly in the tissues must be deduced from this and such storage and saturation of the liver cells was inferred by Mendeloff,

Kramer, Ingelfinger and Bradley (1949) from a study of the changing rate of disappearance of the dye from the peripheral and hepatic venous blood. It has also been questioned whether BRS tests the function of the Kupffer cells or the liver parenchyma (Klein and Levinson, 1933 ; Mills and Dragstedt, 1938).

Casals and Olitsky (1946) point out that the intraperitoneal BRS test resembles a constant intravenous infusion and that a balance is probably struck between absorption rate and rate of elimination. The dose they gave, which was the one we adopted, is large, but they showed that it was innocuous and repeated tests showed no greater retention than the first.

Although eating a meal does not change the rate of elimination of BRS significantly (Mendeloff *et al.*, 1949) starvation was shown by Casals and Olitsky to increase the retention after a few days ; 16 out of 18 mice showed gross retention after five days. Our animals were not starving although they grew at a much reduced rate. Wang and Hegsted (1949) traced a connection between loss of liver nitrogen and loss of power to eliminate BRS, and believed that the BRS retention of protein-deficient diets is due to loss of liver tissue, not to loss of liver function. Flynn and Walshe (1951) found that after excision of the left lobe of a man's liver for carcinoma the BRS elimination was unaffected. The early onset of BRS retention in our rats and its limited increase in degree with time on diet are also arguments against Wang and Hegsted's hypothesis.

The response to the thymol and colloidal gold tests is known to depend on changes in the serum albumin and globulin (Maclagan, 1948) ; these changes and the concentrations of albumin and globulin in the serum are in turn largely dependent on the condition of the parenchymal cells of the liver. The fact that BRS retention occurred so frequently in our rats without necrosis or fibrosis and apart from anything more than an occasional abnormality in other tests of liver function suggests that BRS elimination does depend on something other than the state of the parenchymal cells. Is this possibly impairment of the function of the Kupffer cells as suggested by Mills and Dragsted ?

Himsworth (1949) suggested that one way in which these cystine and tocopherol deficient diets cause necrosis is by depletion of SH groups in the enzyme systems of the liver cells. It is possible that in the growing animal the demand for SH groups is so great that even a few days on diet may lead to deficiencies which are shown up by BRS retention. It requires a stretch of imagination to think that such an enzyme system is directly concerned with BRS elimination but such a system may be an essential in providing the energy required for this process. It is strange, if this is so, that other functions of the liver cell do not seem to be affected.

SUMMARY.

Young rats fed on diets which cause necrosis or fibrosis of the liver very soon become unable to eliminate bromsulphthalein normally, whether necrosis occurs or not.

Such rats very seldom show any change in thymol turbidity or flocculation, colloidal gold reaction, or in serum albumin or globulin concentration.

It is suggested that the failure to eliminate bromsulphthalein is due to impaired activity of the Kupffer cells.

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MICRORADIOGRAPHS AND AUTORADIOGRAPHS OF CORTICAL BONE FROM MONKEYS INJECTED WITH ^{90}Sr.*

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THE macroscopic distribution of ^{90}Sr in the long bones of rabbits has already been described (Jowsey, Rayner, Tutt and Vaughan, 1953). The present paper describes observations on the macroscopic and microscopic distribution of ^{90}Sr in the cortical bone of monkeys.

EXPERIMENTAL PROCEDURE.

Monkeys.

The bones described in this paper were taken from two monkeys, of closely related species, used in a study of strontium metabolism carried out on behalf of the Medical Research Council at the University College of the Gold Coast, Achimota (Edington, Judd and Ward, 1953). One monkey (*Cercopithecus sabeus*) aged approximately four years and therefore not fully grown, died with severe anaemia 24 days after a single injection of 1000 μc. ^{90}Sr/kg. body wt. The second monkey (*Cercopithecus nictitans*) aged at least six years and therefore mature, died 25 days after an injection of 1000 μc. ^{90}Sr/kg. body wt., also with profound anaemia. Death in both animals was attributed to the effects of ^{90}Sr (Edington *et al.*, 1953). The animals were in captivity for a few months only before injection. During this period the calcium content of the diet was 250 mg. Ca *per diem* (Harrison, 1953, personal communication). The calcium content of the diet in the jungle was unlikely to have been higher (Mole, 1953, personal communication). This figure for calcium intake approximates to that given to rabbits on a medium calcium diet (Kidman, Tutt and Vaughan, 1950). The high retention of ^{90}Sr and the pattern of urinary and faecal excretion in the monkeys alone suggests that the calcium content of the diet was low (Kidman *et al.*, 1950; Edington *et al.*, 1953).

Autoradiographs.

Macroscopic.—Macroscopic autoradiographs were prepared as previously described (Kidman, Rayner, Tutt and Vaughan, 1952) using sections approximately 200μ thick. X-ray stripping film supplied by Kodak Ltd. has now been standardized for this purpose.

Microscopic.—To obtain successful microscopic autoradiographs it is necessary to use sections of bone of 50μ or less. These are made by cutting sections as for macroscopic autoradiographs and then grinding them by hand under absolute alcohol on ground glass to approximately 30–50μ as measured by a Baty comparator reading to 1μ. From such sections microradiographs were first made; they were then fixed on a glass slide, coated with a thin layer of celloidin and covered with Kodak fine grain autoradiographic stripping film using the technique of Doniach and Pelc (1950). Cross sections of cortical bone prepared in this way gave satisfactory autoradiographs: those from longitudinal sections of both cortical and trabecular bone often showed artefacts. It is likely that the cross sections are satisfactory because to a large extent they present a smooth surface of approximately the same consistency. The artefacts obtained with longitudinal sections were consistent with pressure effects due to the less even surface obtained owing to the presence of long

* This work was begun on behalf of the Protection Sub-committee of the Medical Research Council's Committee on Medical and Biological Applications of Nuclear Physics.

Haversian systems, and in trabecular bone, of spicules of hard bone alternating with spaces containing soft marrow and large vessels. These artefacts confuse the true autoradiographic picture.

Microradiographs.

The use of the absorption of X-rays to demonstrate areas of different degrees of calcification in cross sections of bone was first suggested by Engstrom (1946). The present microradiographs were made as follows. The section of bone tissue was placed between two layers of " Styrafoil," a cellophane-like substance which does not absorb X-rays, and pressed firmly on to a Kodak maximum resolution plate so that there was good contact between the section and the film. This was then exposed to the beam of X-rays at a distance of about 5 cm. from the target of the X-ray tube. The latter was designed by Ehrenberg and Spear (1951) and manufactured by Hilger and Watts. The focal spot size was always less than 0·1 mm. and the window of the X-ray tube consisted of a 125μ thick beryllium foil. Radiation of 20 Kv. from a chrome target was used and with a tube current of about 400μ amp. the exposure time was of the order of 10 min. The percentage of radiation, after passing through the window, transmitted by various thicknesses of bone was calculated, assuming bone to have the composition given by Engstrom (1946). A graph of this is shown in Fig. 1.

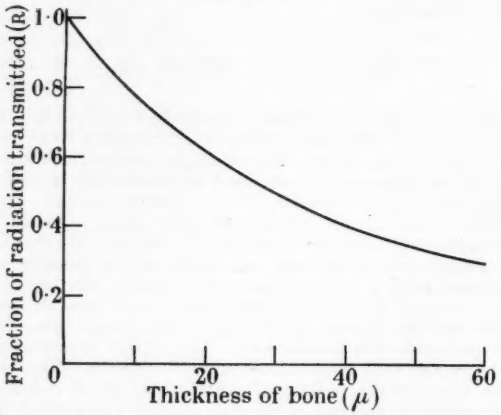


FIG. 1.—The calculated percentage of radiation in roentgens transmitted by various thicknesses of bone expressed in microns.

RESULTS.

Macroscopic Autoradiographs.

Autoradiographs prepared from longitudinal sections of the tibia of the 4-year-old monkey show a diffuse reaction through the spongiosa of the epiphysis and the metaphysis and in cortical bone. There is a strong localised reaction beneath the epiphyseal plate, outlining metaphyseal trabeculae, and in the region of the endosteum throughout the shaft; rare patches of localised reaction occur in the region of the periosteum and there are dots and streaks in cortical bone (Fig. 2). In a cross section of cortical bone the localised reaction is apparent throughout the region of the endosteal bone with rare patches in the region of the periosteal bone. There are well defined speckles of concentrated reaction in cortical bone. These are irregularly distributed and vary in size. The larger areas have a centre showing a less defined reaction (Fig. 3).

Microradiographs.

Monkey 4 years old.—A general view of the appearance of a cross section of the midshaft of the tibia is shown in a low power picture of the whole circumference of the bone (Fig. 4) but considerable magnification is required to appreciate the detailed variation in calcification in different areas. Adjacent to the marrow cavity there is a band of relatively dark, *i.e.*, poorly calcified, bone which sometimes extends all the way along the endosteal surface. This can be seen with higher magnification of an adjacent section of the same bone (Fig. 5) to be clearly defined by a cementing line (Amprino and Engstrom, 1952). Beyond this is an area showing fragments of basic lamellar systems which are highly absorbent, *i.e.*, well calcified, and among which are a variable number of Haversian systems and building sites (Petersen, 1930) or resorption cavities (Petersen, 1930; Amprino, 1951*a*). Both Haversian systems and building sites vary in size, and the absorption of X-rays around them, which is a measure of the degree of calcification, is also variable. This variation may occur round any one system or building site and also from one to another. The bone surrounding one building site may appear well calcified near the rim of the site and less well calcified a little further from the periphery or vice versa, and in some cases the bone surrounding almost the entire site appears well calcified (Fig. 5). The building sites and Haversian canals are largely concentrated at either end of the oval-shaped cross section, large building sites being predominant at the narrower end. A few building sites are, however, apparent in the whole circumference. Externally on the periosteal surface can be distinguished a band of low calcification though in the greater part of its extent it appears better calcified than the corresponding area on the endosteal surface. It is also defined by a series of concentric cementing lines. Throughout the radiographs the lacunae of the osteocytes show up as oval-shaped areas transparent to X-rays. They appear to be arranged concentrically in new bone around building sites and parallel to the cementing lines of endosteal and periosteal bone.

Monkey 6 years old.—The general appearance of the microradiographs of a cross section of the midshaft of the tibia is like that in the younger animal. The building sites and Haversian systems are smaller and probably fewer but the variation in calcification of surrounding bone is considerable and of the same character. In the old monkey there are large areas of highly calcified lamellar bone particularly in the two lateral halves of the oval circumference in which there are no building sites and only extremely small Haversian canals. In adjacent bone the building sites increase until they become numerous at either end of the oval circumference (Fig. 6). Endosteal bone can still be seen to be poorly calcified in most areas while periosteal bone, though defined by arrangement of the osteocytes and cementing lines is, on the whole, well calcified.

Microscopic Autoradiographs.

Autoradiographs were prepared from sections, of which microradiographs had been obtained. They show that the uptake of the radioactive strontium is greatest in areas of low calcification.

No precise quantitative measure of the degrees of greyness on the microradiographs or of the density of the reaction on the autoradiographs has yet been made, but from a qualitative point of view the densest areas of reaction on the auto-

radiograph appear to correspond to the greyest areas on the microradiograph. The areas of least calcification are most commonly found adjacent to the large building sites (Fig. 7 and 8) but do not necessarily extend all round the site. In the monkey which died 24 days after the injection the reaction is found some distance from the edge of the site or cavity (Fig. 8 and 9). There is also some reaction in the poorly calcified areas around typical Haversian systems. The intensity of the reaction corresponds to the degree of greyness on the microradiograph. In the typical small Haversian system the reaction is close to the edge of the canal (Fig. 8). These areas of low calcification which show reaction around building sites are well defined both in the microradiograph and in the histological section by the arrangement of the surrounding osteocytes and by a clear dark line, the cementing line (Amprino and Engstrom, 1952) dividing the area of low calcification from the more highly calcified bone. In areas of high calcification adjacent to building sites no reaction has been observed on the autoradiograph (Fig. 8). There is also reaction above areas of less well calcified endosteal and periosteal bone (Fig. 10 and 11).

DESCRIPTION OF PLATES.

Autoradiographs and microradiographs prepared from longitudinal sections or cross-sections of mid-shaft of tibia of monkeys 4 years and 6 years old dying 24–25 days after a single injection of ^{90}Sr (1000 μc./kg.).

(In order to include as many figures as possible on the available plates, it has been necessary to use an irregular system of numbering.)

FIG. 2.—Macroscopic autoradiograph. Monkey 4 years old. Note (i) generalised diffuse reaction over bone ; (ii) localised reaction (*a*) beneath epiphyseal plate ; (*b*) patchy distribution in region of endosteum, of periosteum and throughout corticalis.

FIG. 3.—Macroscopic autoradiograph (" negative print "). Monkey 4 years old, from adjacent section to Fig. 4. Note (*a*) diffuse reaction throughout bone ; (*b*) localised reaction ; (i) irregular in intensity in endosteal region ; (ii) trace only in periosteal region ; (iii) small round areas in corticalis of high intensity with centres, in some cases of reduced intensity. × 6.

FIG. 4.—Microradiograph (section 50 μ). Note (i) Haversian systems ; (ii) building sites and resorption cavities ; (iii) variations in calcification around building sites and in endosteal bone and periosteal bone. × 6.

FIG. 5.—Microradiograph. Monkey 4 years old (section adjacent to that from which Fig. 4 prepared). Note (i) low calcification of endosteal bone (lower edge of section) ; (ii) less well marked calcification of periosteal bone (upper edge of section) ; (iii) building sites ; (iv) Haversian canals ; (v) variable degrees of calcification around both (iii) and (iv) ; (vi) high calcification of lamellar bone outside zone of building sites. × 80.

FIG. 6.—Microradiograph. Monkey 6 years old. Note (i) low calcification of endosteal bone ; (ii) small Haversian canals with little variation in calcification in highly calcified lamellar bone ; (iii) small building sites in one area only. × 40.

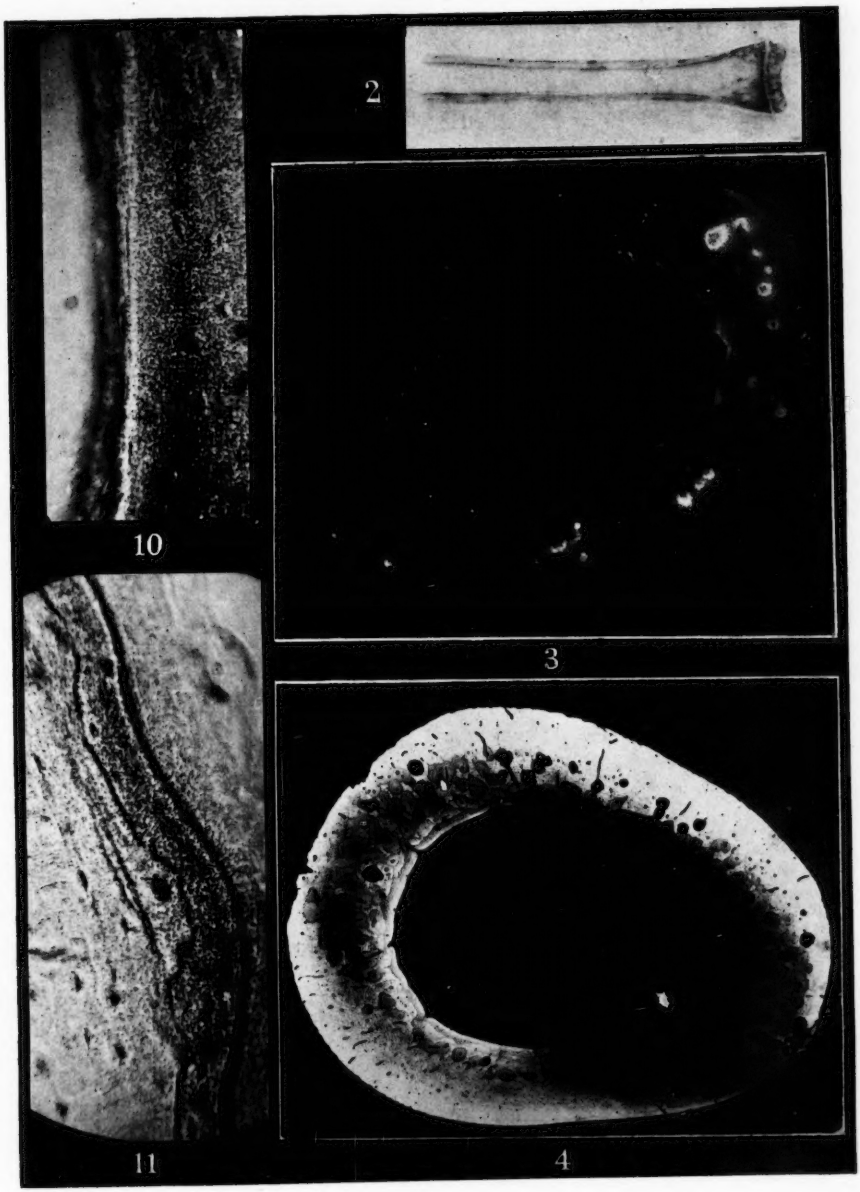
FIG. 7.—Microradiograph. Monkey 4 years old. Note (i) low calcification endosteal bone (upper edge of section) ; (ii) building sites ; (iii) Haversian canals ; (iv) variable degrees of calcification around both (ii) and (iii). × 80.

FIG. 8.—Autoradiograph left on section from which Fig. 7 prepared so that detail of bone structure is seen through the stripping film emulsion. Note (i) reaction over areas of low calcification around building sites and Haversian canals and over endosteal bone upper edge of section. Reaction appears as a black or greyish area depending on its intensity. × 80.

FIG. 9.—Autoradiograph left on cross section. Note (i) detail of reaction around building site. There is an area of dense black granules at the upper part of the building site some distance from the edge with greater scattering of the granules on either side of this dense granulation. The cavity itself is clear. There are a few lacunae. × 300.

FIG. 10.—Autoradiograph left on section. Note (i) detail of reaction over endosteal bone. There is an area of dense black granules a little away from the inner surface of the bone with a diffuse scatter of granules on either side. The lacunae of the osteocytes are seen. × 300.

FIG. 11.—Autoradiograph left on section. Note (i) detail of reaction over periosteal bone. There is an area of dense black granules a little away from the outer surface of the bone with a diffuse scatter of granules on either side. Cementing lines and lacunae of the osteocytes are clearly seen. × 300.



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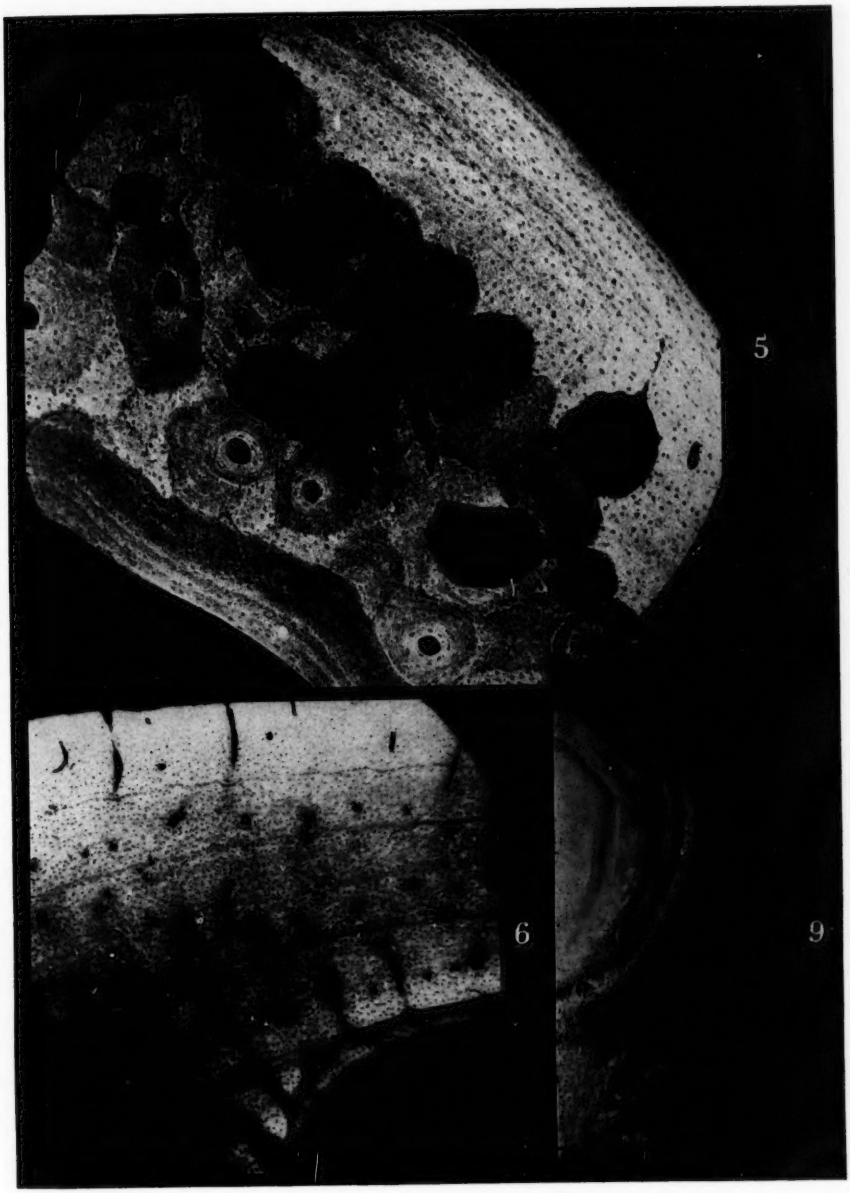

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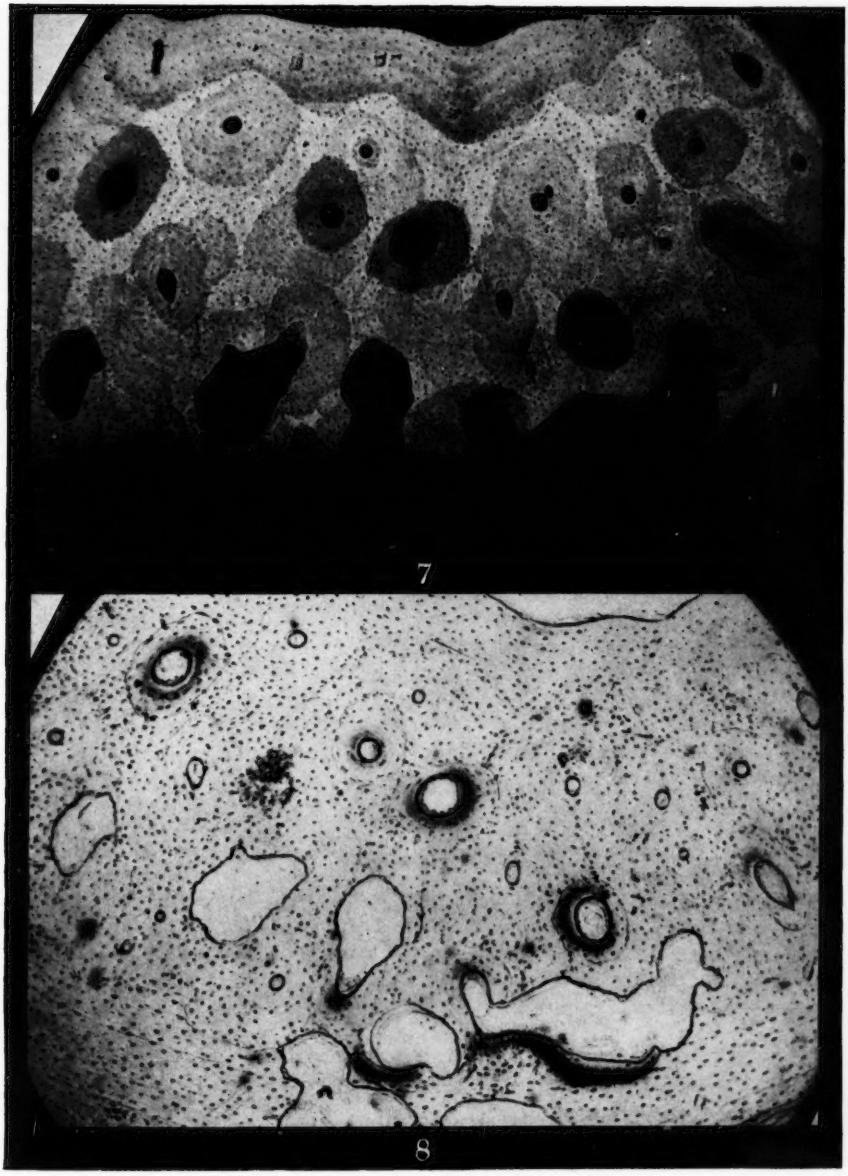

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DISCUSSION.

The processes of resorption and apposition in cortical bone are continuous throughout life (Petersen, 1930; Amprino 1951*a*, *b*; Amprino and Engstrom, 1952) though reconstruction in the diaphysis is much more marked in long-lived animals, particularly carnivora, than in short-lived (Amprino, 1951*b*). Petersen (1930) has described and illustrated the histological changes occurring in the cortical bone of normal men and children who met with sudden death when healthy. Reconstruction is dependent upon changes occurring in areas he calls "Bauplätze" or building sites. In such areas associated either with a Haversian system or a Volkmann's canal, osteoclastic activity begins leading to resorption of bone and formation of apparent holes. Soft tissue containing blood vessels present in such a building site, as resorption goes forward, tends to fall out of ground sections leaving only a hole unless great care is taken. Subsequently, in the same area, often simultaneously with osteoclastic resorption, apposition by osteoblasts will begin and finally the cavity is almost entirely filled with new bone leaving a new Haversian system. This description of Petersen fits well with what is seen in the present autoradiographs and microradiographs of cross sections. In the latter, the centre of a building site which is composed of soft tissue always appears as a hole. These holes vary widely in shape and size. There may be three broad types: (i) surrounded with highly calcified bone in which it can be presumed that osteoclastic activity only is present (Bloom, Bloom and McLean, 1941); (ii) surrounded with poorly calcified bone in which osteoblastic activity only is present; (iii) surrounded with some highly calcified and some poorly calcified bone in which both osteoblastic and osteoclastic activity is going forward. In the autoradiographs building sites of type (i) show no reaction around them, building sites of type (ii) show heavy reaction, and building sites of type (iii) show reaction in one part and not in another. Between these three groups various intermediate pictures are found. The uptake of strontium therefore in cortical bone appears to follow the same biological pattern as the uptake in bone beneath the epiphyseal cartilage, *i.e.*, it occurs in areas of active bone growth (Kidman *et al.*, 1952).

In the monkey that died 24 days after injection the area of bone showing a dense reaction in the autoradiograph is separated from the hole of the building site by a narrow rim of bone showing considerably less reaction. Presumably bone containing strontium was laid down immediately following injection when the level of strontium in the blood was high, and this was subsequently displaced by bone laid down when the blood level was low and therefore the uptake was insignificant. The pattern of growth in older animals in cortical bone around building sites is in other words the same as the pattern seen in epiphyseal bone in younger animals (Kidman *et al.*, 1952).

In the bones of monkeys where the building sites are large the speckled appearance due to uneven distribution of radioactive strontium around unevenly calcified building sites is seen in the geographical autoradiographs of animals not fully grown. In the bones of rabbits where building sites are smaller and therefore closer together the uneven calcification is not apparent in young animals but becomes obvious in the geographical autoradiographs of old animals where there are fewer scattered building sites (Kidman *et al.*, 1952; Jowsey, Owen and Vaughan, to be published). It should be remembered that the monkeys had

only a short period of controlled calcium intake and a high retention of strontium which may have affected the intensity of reactions in the picture described.

A similar patchy uptake of radioactive calcium and radioactive phosphorus associated with building sites has been demonstrated by *in vitro* techniques in human bone (Amprino, 1951*b*, 1952), while the uptake of radioactive phosphorus in the same sites in dog bone has been found following injection (Engfeldt, Engstrom and Zetterstrom, 1952). Since there is no reason to suppose that the deposition of radium will not follow the same pattern as that of calcium and strontium, it is possible that the occurrence of " radium hot spots " in cortical bone (Aub, Evans, Hempelmann and Martland, 1952) may be dependent on deposition in building sites where osteoblastic activity predominated at the time of ingestion or at the time when radium was released by resorption from other sites and available for secondary uptake.

The technique of examining the same or adjacent sections of bone by microradiography, autoradiography and histochemistry following injection of radioactive isotopes should throw considerable light on the physiology of bone. It is, however, clear that for a full understanding of the fate of any one element taken up by bone, observations over a long period of time will be required together with a detailed knowledge of the normal structure of bone using these combined techniques. Work on rabbits is at present in progress along these lines. Preliminary observations suggest that the uptake of radioactive strontium in cortical bone in these animals follows the same pattern as that described in monkeys.

SUMMARY.

The appearances in microradiographs and autoradiographs prepared from the same cross section of cortical bone from the tibia of monkeys following the injection of ^{90}Sr are described.

^{90}Sr is taken up in areas of low calcification in endosteal and periosteal bone and around building sites where apposition is taking place. At the microscopic level as at the macroscopic level, concentration of ^{90}Sr occurs only in areas of bone growth. Such uptake is extremely uneven in distribution.

It is suggested that the uptake of ^{90}Sr around active building sites may account for the occurrence of " radium hot spots."

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THE POSITION OF TAYLOR'S 1233 STRAIN OF VIRUS IN THE HUMAN RED CELL RECEPTOR GRADIENT.*

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SINCE the observation of haemagglutination by influenza virus (Hirst, 1941) red cell suspensions have been used extensively in the study of influenza viruses. If red cells and influenza virus are mixed, the virus attaches itself to the red cell surface and agglutination results; then, after a period, the virus elutes from the red cell surface (McClelland and Hare, 1941; Hirst, 1942) while retaining all the properties of the original particle. The red cell, however, is altered so that a fresh sample of the same influenza strain will not agglutinate the treated red cells. This action has been compared with the action of an enzyme on its substrate.

Burnet, McCrea and Stone (1946) have prepared a receptor-destroying enzyme (RDE) from a cell-free filtrate of *Vibrio cholerae* which has similar enzymic activity to that attributed to influenza virus. RDE alters the red cell surface in such a way that the cells are no longer agglutinated by the viruses of the mumps, Newcastle disease, influenza group (M.N.I.). This is seen most readily when high concentrations of enzyme are used. When varying amounts of RDE are used to treat red cells, the ability of different strains to agglutinate the treated cells falls into a series. The "receptor gradient" established by Burnet *et al.* (1946) and based on the ability of the virus to agglutinate treated human cells was in the order: Newcastle disease, MEL, WSE, LEE, Mumps, SW and MIL B. The receptors for Newcastle disease were the first to be destroyed by RDE.

Taylor's 1233 strain of virus (Taylor, 1949) was not available when the gradient was determined. The 1233 virus was isolated from a patient with mild respiratory symptoms. Although serologically distinct from any member of the M.N.I. group, Taylor's 1233 strain has several properties common to it. It agglutinates human and fowl cells, and then rapidly elutes from them. The virus particles after elution apparently retain their original properties but the red cells are no longer agglutinated by fresh samples of the virus. This suggested that the virus may be another member of the M.N.I. group.

Subsequent work (Hirst, 1950) in which fowl cells and RDE were used failed to indicate that 1233 virus reacted with the same receptors as members of the M.N.I. group. Hirst, however, considered that there was a relationship between Newcastle disease virus (NDV) and the 1233 virus since fowl erythrocytes, after treatment with 1233, did not agglutinate to full titre with NDV.

A preliminary paper (White, 1952) reported that human erythrocytes, after treatment with RDE, were not agglutinated by 1233 virus. Taylor's 1233 virus was situated between WSE and LEE in the human red cell gradient established by RDE treatment. This paper extends the subject of the previous publication.

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MATERIALS AND METHODS.

Solutions.—Normal saline : 0·85 per cent sodium chloride in distilled water.

Citrate saline : 2 per cent sodium citrate in normal saline.

Calcium acetate saline : 0·1 per cent calcium chloride, 1·23 per cent sodium acetate and 0·5 per cent sodium chloride. Sufficient acetic acid (approx. 0·026 M) was added to buffer the solution at pH 6·2.

Erythrocytes.—Human red cells were obtained from group O donors. Fowl red cells were taken from the wing vein of a White Leghorn fowl.

The red cells were collected in an equal volume of citrate saline, washed three times in normal saline and stored at 4° until used. For haemagglutinin titrations 1 per cent suspensions of the packed cells were prepared in normal saline.

Viruses.—The egg-adapted strains of the following viruses described by Burnet *et al* (1946) were used :

NDV, MEL, WSE, LEE, SW and MIL B.

1233, the strain of virus isolated in the U.S.A. in 1947 (Taylor, 1949).

For the cultivation of viruses in chick embryos the techniques described by Beveridge and Burnet (1946) were followed. Seed virus was stored at − 20° as allantoic fluids to which 25 per cent glycerol was added. Strains of type A and type B influenza virus were diluted 10^{-2} and NDV was diluted 10^{-6} for inoculation into the allantoic cavity of 10 day embryos. The allantoic fluids were harvested after incubation for 2 days at 36°. Taylor's 1233 virus, diluted 10^{-4}, was inoculated into the amniotic cavity of 9–11 day embryos and the allantoic and amniotic fluids were harvested separately after 2–3 days at 36°. Growth of 1233 virus could only be successfully maintained by amniotic inoculation. However, the allantoic fluid from these eggs frequently contained considerable amounts of 1233 virus and infected allantoic fluids have been largely used as a source of virus for *in vitro* experiments.

Titration of virus haemagglutinin.—The technique is that currently used in the Hall Institute (Burnet, Beveridge, Bull and Clark, 1942) and is based on the pattern of red cells deposited in a tube with a hemispherical bottom.

Receptor destroying enzyme (RDE) of *V. cholerae* was used as a crude extract (Burnet and Stone, 1947) or purified (Ada and French, 1950). Both these preparations were kindly supplied by Mr. E. L. French and Mr. G. L. Ada of the Walter and Eliza Hall Institute. RDE was titrated as described by Burnet and Stone (1947).

Treatment of red cells with RDE.—RDE was diluted in calcium acetate saline through a range of dilutions from 100 to 0·75 units per volume (0·25 ml.). A control tube was prepared by boiling for 15 min. a solution containing 100 units per volume. Ten per cent by volume of washed packed human cells was added to each tube of RDE dilution including the control tube and the mixtures were incubated for 2 hr. at 37°. At the end of this period, further RDE action was prevented by the addition of an equal volume of citrate saline to the cell suspension. The suspensions were then centrifuged, washed once in citrate saline and twice in normal saline.

Treatment of red cells with viruses.—Sufficient undiluted virus-infected allantoic fluid was added to packed human cells to give a 10 per cent cell suspension. The mixture was held at 37° for 4 hr. The cells were then washed in three changes of normal saline and tested for stability. Cells unstable (agglutinating in saline) after this period were, with the exception of those treated with NDV, mixed with immune ferret sera diluted 1 in 50 and held at 4° overnight. Cells treated with NDV but still unstable were re-suspended in normal saline and incubated for a further 4 hr. at 37°. After the second treatment, the cells were washed in three changes of saline and tested for stability. In all cases the red cell suspension was then stable in saline.

RESULTS.

Destruction of human red cell receptors by Taylor's 1233 virus.

Human cells were treated with 1233 virus in the following manner. Allantoic fluid infected with 1233 virus was mixed with human cells to give a 10 per cent suspension. After 4 hr. at 37°, the red cells were washed in three changes of

normal saline and re-suspended as a 1 per cent suspension in normal saline. As a control, red cells were treated in a like manner with normal 13-day allantoic fluid.

The treated red cells were used to titrate the haemagglutinin of various influenza viruses (see Table I).

TABLE I.—*Agglutination of Human Red Cells after Treatment with Taylor's 1233 Virus.*

Cells treated with—	Titre to which cells were agglutinated by virus.						
	1233.	NDV.	MEL.	WSE.	LEE.	SW.	MIL B.
Taylor's 1233 virus . . .	<1	80	480	320	640	240	30
Normal allantoic fluid . . .	160	160	480	240	640	320	40

In all cases, the 1233-treated human red cells were completely resistant to agglutination by any concentration of 1233 virus tested. However, using 1233-treated cells, the apparent titre of NDV was reduced. The NDV haemagglutinin titre against 1233 treated cells, when compared with that obtained with normal untreated cells in 14 tests varied between 25 and 120 per cent of the control with a mean of 60 per cent. There was no significant reduction of the haemagglutinin titre of any type A or type B virus strains with the 1233-treated cells.

Destruction of 1233 *receptors on human cells by treatment with RDE.*

(a) *Prevention of haemagglutination by* 1233 *and other viruses.*—Successive batches of human cells were treated with falling dilutions of RDE as described above. The concentrations of RDE used were in the range 100 to 0·75 units per 0·25 ml. Each batch of treated cells was used to titrate the haemagglutinin of a number of influenza viruses, of NDV and of 1233. Typical results with crude RDE are given in Table II and the results with purified RDE in Table III.

TABLE II.—*Haemagglutination Titre of Influenza Viruses using Human Red Cells Treated with Crude RDE.*

Virus suspension.	RDE units per 0·25 ml. in preparation used to treat cells.								Boiled RDE 100 units/ 0·25 ml. (control).
	100.	50.	25.	12.	6.	3.	1·5.	0·75.	
1233 virus amniotic fluid . .	<10	<10	<10	320	320	640	800	800	800
1233 virus allantoic fluid . .	<10	<10	<10	80	160	240	320	320	240
NDV	—	—	—	<10	<10	10	20	40	40
MEL	<10	<10	<10	<10	80	640	640	640	640
WSE	<10	<10	<10	20	80	160	240	160	160
LEE	<10	40	160	480	480	—	—	—	320

— = titre of haemagglutinin was not determined.

These figures provide *prima facie* evidence that both crude and purified RDE destroyed the receptors of human red cells for 1233 virus. Furthermore, in the gradient so determined with RDE, 1233 occupied a position between WSE and LEE viruses.

(b) *Prevention of adsorption of* 1233 *virus.*—One batch of human red cells was treated as above with a final concentration of 100 units per 0·25 ml. of RDE for 2 hr. at 37° and the cells washed in one change of citrate and two changes of

TABLE III.—*Haemagglutination Titre of Influenza Viruses using Human Red Cells Treated with Purified RDE.*

Virus suspension.	RDE units per 0·25 ml. in preparation used to treat cells.							Boiled RDE 100 units/ 0·25 ml. (control).
	100.	50.	25.	12.	6.	3·0.	1·5.	
1233 virus amniotic fluid	<10	<10	<10	80	320	2000	2000	2000
1233 virus allantoic fluid	—	—	<10	<10	40	50	80	100
MEL	—	<10	<10	<10	<10	20	160	480
WSE	<10	<10	<10	<10	120	320	480	480
LEE	<10	<10	10	160	480	800	800	800

— = titre of haemagglutinin not determined.

saline. A volume of 0·1 ml. of these packed cells was added to 1 ml. of allantoic fluid containing 80 A.D. of 1233 virus per ml. The mixture was held at 4° for 15 min. and then centrifuged. The titre of haemagglutinin in the supernatant was determined using fresh 1 per cent human cells. This experiment was controlled by the substitution of boiled for active RDE.

Whereas in the control, the titre of virus was reduced from 80 A.D./ml. to less than 8 A.D./ml., in the test the titre of the supernatant remained at 80 A.D./ml. The treated cells had not absorbed haemagglutinin after 15 min. at 4°.

This experiment was repeated with progressively shorter time intervals down to 2 min. The findings were the same as when a 15 min. interval was used. Control cells absorbed at least 50 per cent of 1233 haemagglutinin within 2 min. but RDE-treated cells absorbed none.

Failure of Taylor's 1233 *virus to agglutinate cells treated with NDV and with swine influenza virus.*

The viruses NDV, LEE, MEL, and SW were used to treat different batches of human cells as described above. Cells so prepared were washed in saline, re-suspended as 1 per cent suspensions in normal saline and used to titrate the haemagglutinin of 1233 and of the various M.N.I. viruses (Table IV).

TABLE IV.—*Agglutinability of Human Red Cells after Treatment with Various Viruses.*

Cells treated with—	Titre to which cells were agglutinated by virus.				
	1233.	NDV.	MEL.	LEE.	SW.
Normal allantoic fluid	160	480	1280	640	160
NDV	30	<10	<10	640	120
MEL	160	<10	<10	640	160
LEE	160	<10	<10	560	120
SW	<10	<10	<10	<10	20

The results fall into two groups. First, treatment of cells with LEE did not affect the apparent titre of LEE or 1233. Similarly, the treatment of cells with MEL did not affect the apparent titre of 1233. MEL-treated cells were not agglutinated by MEL.

Secondly, both SW and NDV treatment of cells reduced the agglutinability of the cells by 1233. SW-treated cells were agglutinated only by a concentration of 1233 many times greater than that required to agglutinate the control. However, the treated cells were still agglutinated by SW virus to a titre approximately

12 per cent of that with the control cells. Similarly NDV treated cells were agglutinated only by a concentration of 1233 at least five times that required to agglutinate control cells. The NDV-treated cells were not agglutinated by NDV.

DISCUSSION.

Taylor (1949) isolated the 1233 strain of virus from a case, clinically diagnosed as influenza, by inoculation into the amniotic cavity of the chick embryo. It agglutinated fowl cells.

No serological relationship between 1233 and members of the M.N.I. group of viruses has been found. By dual infection of ferrets, Taylor (1951) has established that there is no antigenic relationship between 1233 virus and various type A and B influenza strains.

From a patient with mild respiratory symptoms Francis isolated a virus (J.J.) which also agglutinated fowl cells (Francis, Quilligan and Minuse, 1950). Sera from this patient showed a significant rise in antibody to both J.J. and 1233 virus by neutralization in eggs, complement fixation and anti-haemagglutinin techniques. Francis considered that J.J. virus was similar to Taylor's 1233 virus and distinct from any type A or B influenza virus and he proposed that these two viruses be classified as type C influenza virus.

Hirst (1950) studied the relationship of 1233 virus to members of the M.N.I. group. In the chick embryo 1233 impeded the growth of LEE virus and in mice it interfered with the growth of Western equine encephalomyelitis virus. The fact that certain influenza strains were also known to show a similar interference phenomenon (Henle and Henle, 1945) provided a hint of a relationship between 1233 and influenza viruses. However Hirst was unable to show any relationship between the fowl red cell receptors of 1233 and of the M.N.I. group and considered that 1233 virus was acting on different receptors.

We report that RDE treatment of human red cells destroys the red cell receptors for Taylor's 1233 virus which falls between WSE and LEE in the human red cell receptor gradient. The haemagglutinin titre of 1233 is markedly reduced if cells are previously treated with NDV or SW. The haemagglutinin titre is not affected on the other hand, by the prior interaction of the red cells with LEE or MEL virus . This suggests that 1233 is attached to a cell component which is altered only by SW, NDV and RDE. It appears that LEE and MEL do not act to a significant extent on the same component as 1233.

Stone and Ada (1950), from a comparison of the changes in electrophoretic mobility (EPM) with loss of agglutinability by viruses of the gradient series, concluded that there were two groups of cell surface components concerned with determining the EPM and susceptibility to enzymic destruction. One set was responsible for agglutination by a majority of the M.N.I. viruses and its components were susceptible to enzymic destruction by these. The receptor gradient depended essentially on the variable ability of different strains to react with these receptors. The second set of receptors could be destroyed by RDE and to a considerable extent by SW and NDV viruses. There was no unequivocal evidence that this group of receptors played any part in binding NDV or SW to the cell surface.

The present results strongly suggest that 1233 reacts only with the second set of receptors. Stone and Ada (1952) showed that RDE acts progressively on both the first and second series. The position of 1233 in the gradient between WSE and LEE presumably means that by the time RDE has destroyed the reactivity of

the first sequence to the point corresponding to loss of agglutinability by WSE, it has also almost destroyed the reactivity of the second sequence of receptors for 1233. It may well be that 1233 is the only virus so far tested that is capable of being bound to the second type of receptor in such a manner as to produce haemagglutination. Its action in diminishing the agglutinating power of NDV suggests that it may also have a very limited power to destroy some receptors of the first sequence.

Taylor's 1233 virus fulfils the following characteristics of members of the M.N.I. group of viruses:

(*a*) Isolation from human cases.
(*b*) Ready growth in chick embryo.
(*c*) Haemagglutination of and elution from red cell surfaces.
(*d*) Destruction of its appropriate receptors on human red cells by RDE and certain members of the M.N.I. group.

Taylor's 1233 virus is, therefore, believed to be a member of the M.N.I. group of viruses. We agree with Francis that it should be classified as type C influenza virus.

SUMMARY.

The haemagglutinating properties of Taylor's 1233 virus have been investigated in relation to members of the mumps, Newcastle disease, influenza (M.N.I.) group of viruses.

The human red cell receptors for 1233 are destroyed by the action of the receptor destroying enzyme (RDE) of *V. cholerae*, swine influenza and Newcastle disease viruses but not by the LEE or MEL strains of influenza virus. An explanation for these findings is suggested.

The similarity of the properties of Taylor's 1233 virus and those of the M.N.I. group indicates that 1233 virus is a member of the group. These findings support the classification by Francis of 1233 as a strain of type C influenza virus.

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INFLAMMATION AS INFLUENCED BY A TOPICAL BLOCKADE OF THE RETICULO-ENDOTHELIAL SYSTEM:

AN EXAMPLE OF "LOCAL CROSSED SENSITISATION."

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STUDIES of the resistance of connective tissue to topically applied irritants are difficult to interpret quantitatively because, with existing laboratory techniques, a strictly reproducible area of contact between tissue and irritant is unobtainable. Injected substances tend to be distributed irregularly, according to the injection pressure, the configuration of the connective tissue layer penetrated, the extent of incidental trauma, etc. These variables are eliminated if the irritating agent is directly applied to a standardised wound surface, but here the prevention of infection is difficult and some of the substance is liable to be lost before it can act on the tissues. These sources of error are particularly disturbing when it is desired to expose the same connective tissue surface repeatedly to measured amounts of irritants.

To remedy these shortcomings, the so-called "granuloma pouch" technique was designed and found especially useful in defining the effect of a topical "blockade of the reticulo-endothelial system" on the local actions of irritants. This method consists of producing a sterile connective tissue sac by the subcutaneous injection of a measured amount of air. Irritants then introduced into this cavity are necessarily placed in permanent contact with the same tissue surface.

A systemic blockade of the reticulo-endothelial system, induced by the intravenous injection of particulate substances, diminishes resistance to various pathogens. An excellent review of the extensive literature of this subject is given by Cameron (1952). But because of the above-mentioned technical difficulties little is known about the direct local effects of such a "blockade" on tissue reactions to irritants subsequently administered to the same area, under conditions which eliminate possible complicating systemic reactions (*e.g.*, those due to secondary humoral changes or to the blockade of macrophages in the spleen, bone-marrow and liver).

Avery and Rigdon (1939) studied this by injecting India ink intradermally in rabbits and then introducing pneumococci either into or around the stained area. They found that, if the bacteria are injected just outside the pre-treated region, they cause an inflammation which avoids the ink-storing area. From this they concluded "that skin injected with India ink does not permit the infiltration of bacteria, oedema fluid, and leucocytes from an advancing inflammation." However, when the pneumococci were injected directly into the ink-stained tissue, then the inflammation spread readily thence into surrounding tissue. They

thought that " this is probably due in part to the disruption caused by the pressure of the subsequent injection."

It is noteworthy that if the pneumococci were injected outside the pre-treated area 24 hours after the India ink injection, the refractoriness of the stained tissue was less pronounced than if the time interval between the two injections was longer. It is probable that after a long interval the inflammation caused by the pneumococci actually did not come into any direct contact with the cells which had phagocytosed the ink particles. This is implied by the photomicrographs given by these authors, as well as by many incidental observations made in this laboratory on rats given subcutaneous injections of India ink. Here, within a week or two, the stained injection site is invariably surrounded by a thick layer of dense connective tissue. It is very likely, therefore, that the spread of the bacterial inflammation into the pre-treated area was stopped by such a fibrous capsule, and was unrelated to the presence of ink-laden phagocytes. On the other hand, we agree that it is difficult to interpret the findings after introduction of the pneumococci into the centre of the ink-stained area. Here the bacteria are presumably disseminated in an entirely random manner ; in fact, the pressure of the injection may well disrupt such hardened and brittle nodules, so as to permit the immediate escape of pneumococci into the surroundings.

Earlier experiments performed in this Institute, using the granuloma pouch technique, have shown that an inflammatory granuloma protects against certain irritants, while others penetrate and destroy it even more readily than they attack normal connective tissue. Thus, if a granuloma pouch is produced by pre-treatment with dilute croton oil, its wall is resistant to the subsequent introduction into the central cavity of large doses of formic acid, hypertonic sodium chloride, mustard oil, sodium hydroxide, hydrochloric acid, trypsin, pepsin, ox bile and even boiling water (Selye, 1953*a*, *b*, *c*). This phenomenon was named " topical crossed resistance." On the other hand, such a granuloma pouch was even less resistant to the destructive action of formaldehyde than connective tissue not pre-treated (Selye, 1953*b*).

Pre-treatment with any of these agents induced " specific resistance " to subsequent introduction into the pouch of higher concentrations or greater amounts of the same irritant (Selye, 1953*d*, *e*).

There is no reason to believe that these induced changes in topical tissue resistance are more directly related to alterations in the activities of the phagocytes than to other manifestations of the inflammatory process, such as fibroblast proliferation, exudation or hyperaemia. Yet, in view of the part played by the reticulo-endothelial system in defence, the experiments now to be described were performed with a typical phagocyte " blocking agent," India ink. This material is known to cause no serious tissue injury, although it is rapidly taken up by phagocytes, presumably " blocking " their functions. We therefore used the granuloma pouch method to try to decide whether saturation of a limited connective tissue area with India ink particles would alter its resistance to a subsequent exposure to an irritant such as croton oil.

EXPERIMENTAL.

The " granuloma pouch " technique.

This has already been described together with its principal applications in the study of inflammation and wound-healing (Selye, 1953*b*, *d*, *e*, *f* ; Selye and Horava, 1952). In the

present work the method was used as follows : The dorsal hair of rats was removed with fine electric clippers. Under ether anaesthesia, after cleaning the skin with alcohol and metaphen, 25 ml. of air was injected into the loose connective tissue between the shoulder blades with a No. 27 needle. This was immediately followed by the injection of the substance or substances to be tested, the air being first injected with a large syringe and the needle left *in situ* for the subsequent injection of the material to be tested.

Because of the great natural resistance of the rat to infection, strict sterility precautions were not enforced, but syringes, needles and the solutions to be injected were sterilised by boiling, except the croton oil ; this is difficult to sterilise without partial inactivation, and since it causes much inflammation, which in itself tends to combat infection, sterilisation was not thought indispensable. In fact, none of the rats showed any sign of infection.

Procedure in the first experimental series.

The first series of experiments was performed on 50 female albino Wistar rats, weighing 120–140 g. These were subdivided into 5 groups of 10 rats each, and treated as outlined in Table I.

The India ink suspension consisted of " Higgins' American India Ink " diluted 1/5 with 0·85 per cent NaCl solution.

Croton oil was used as a 1 per cent solution in corn oil.

The five groups were treated as follows :

I. India ink (2 ml.) was injected immediately after producing a sac with 25 ml. of air. While the rats were still under anaesthesia, the ink was evenly distributed over the inner surface of the sac by simple shaking. This had to be done rapidly after the injection of the ink, since the fluid is absorbed within a few minutes, after which even distribution is no longer possible.

II. Treatment with India ink was the same as in Group I but, after absorption of the suspension fluid, 1 ml. croton oil solution was injected.

III. Physiological saline (2 ml.) was injected instead of India ink ; followed by croton oil as in Group II.

IV. An injection of 4 ml. India ink only was given to determine whether any difference in the response of Groups I and II could be duplicated by a mere increase in the quantity of ink.

V. An injection of 2 ml. croton oil solution only was given to determine whether any difference in the response between Groups II and III could be duplicated by doubling the dose of croton oil.

The animals were observed for 48 hours, macroscopically visible necrosis of the covering skin being noted ; they were then killed. The pouch was then dissected, together with the covering skin, and fixed in Susa solution, after opening it to permit better penetration. Paraffin sections were stained with haematoxylin and eosin.

Procedure in the second experimental series.

The second experiment differed from the first only in that the India ink was not injected into the cavity itself, but into a part of the lining connective tissue.

After some preliminary experiments, it was found that this can be accomplished best by injecting 0·2 ml. of the same (20 per cent) India ink suspension subcutaneously before injecting the air. Air (25 ml.) was then injected as in the first experiment. The entire ink-stained tissue was then invariably lifted up above the air sac, since the infiltrated tissue is more adherent to the skin than to the underlying muscle. Thus a circumscribed area—of what has now become the pouch lining below the skin—is saturated with carbon particles.

Here, again, controls were given saline, as well as a double dose of India ink (into the wall), and croton oil (into the cavity), as specified in Table II. After noting visible necrosis, the animals were killed 48 hours following the injections, and the tissues taken for histological study.

RESULTS.

Macroscopic Findings.

The results of the first experimental series are summarised in Table I. India ink alone caused no evident necrosis (Group I), while the same amount of ink,

followed by treatment with croton oil, caused evident necrosis in 90 per cent of animals. The mere injection of fluid was not responsible for this, since the same volume of saline caused no such sensitisation to subsequent treatment with croton oil, and none of the animals so treated (Group III) showed necrosis.

TABLE I.—*Effect of India Ink Injected into a Granuloma Pouch Cavity on the Response to Immediately Subsequent Treatment with Croton Oil.*

Group.	Treatment.	Incidence of necrosis (per cent).
I	Ink, 2 ml., in cavity	0
II	Ink, 2 ml. + croton oil, 1 ml., in cavity	90
III	NaCl solution, 2 ml. + croton oil, 1 ml., in cavity	0
IV	Ink, 4 ml., in cavity	0
V	Croton oil, 2 ml., in cavity	10

These observations did not suffice to prove true " crossed sensitisation "—*i.e.*, a decrease in tissue resistance induced by one agent (India ink) against another agent (croton oil)—since in Group II a greater amount of potentially irritating material was introduced than either in Group I or in Group III. However, doubling the dose of India ink (Group IV) caused no necrosis, while doubling the dose of croton oil (Group V) caused some evidence of necrosis in one animal only.

We therefore felt justified in concluding that saturation of connective tissue with India ink diminishes its capacity to withstand the damaging effect of subsequently applied croton oil. Indeed, the otherwise almost inert ink particles were more efficacious than an additional dose of croton oil, which in itself is much more irritating.

The results of the second experimental series are stated in Table II. They were strikingly similar to those in the first. Local " blockade " of the phagocyte system diminished the resistance of the tissue to subsequent treatment with croton oil. Significantly, the necrosis always occurred wherever the ink spot happened to be under the surface of the skin. Here again doubling the dose of India ink, or of croton oil, failed to duplicate the intense tissue damage seen in the animals receiving croton oil after the ink injection (Group II).

TABLE II.—*Effect of India Ink Introduced into the Wall of a Granuloma Pouch on the Response to Immediately Subsequent Injection of Croton Oil into the Cavity.*

Group.	Treatment.	Incidence of necrosis (per cent).
I	Ink, 0·2 ml., in wall	0
II	Ink, 0·2 ml., in wall + croton oil, 1 ml., in cavity	90
III	NaCl solution, 0·2 ml., in wall + croton oil, 1 ml., in cavity	0
IV	Ink, 0·4 ml., in wall	0
V	Croton oil, 2 ml., in cavity	10

Microscopic Findings.

Histological examination confirmed the macroscopic findings, as regards the occurrence of necrosis, but it revealed additional features of the reaction.

If India ink alone is introduced into the granuloma pouch it distributes itself evenly on the inner surface, and forms a clearly drawn black line in section. It never causes suppuration, although it may attract a few polymorphonuclear leukocytes (Fig. 1).

When the injection of ink into the cavity is followed by croton oil, there first develops a rather massive suppuration in the tissue outside the ink-stained margin (Fig. 2). This is rapidly followed by a disintegration of the polymorphonuclear leukocytes in this layer, with necrosis of the adjacent tissues (Fig. 3).

Croton oil alone produces a connective tissue response, characterised primarily by intense proliferation of particularly large and basophilic fibroblasts, arranged around newly formed capillaries in a rather characteristic manner (Fig. 4), as described earlier (Selye, 1953*d*).

Since neither ink alone, nor croton oil alone, duplicates the intensely suppurating and necrotising reaction caused by the combination of these irritants, it is reasonable to assume that this type of response is due to an interaction between the two agents.

If India ink is introduced directly into the wall of the granuloma pouch, it causes some suppuration, but this is limited to the site of injection. It may well be due to the combined action of the carbon particles and the trauma induced by

DESCRIPTION OF PLATES.

All photographs are of regions just outside the inner surface of that part of the granuloma pouch which is adherent to the skin, the free surface being near the right margin.

FIG. 1.—Rat from Group I of the first experimental series (2 ml. of India ink in cavity). The carbon particles are evenly distributed along the inner surface and form a clearly drawn black line. There is no sign of suppuration and the surrounding connective tissue shows only a slight degree of proliferation. The underlying dermal muscle is normal.

FIG. 2.—Rat from Group II of the first experimental series (2 ml. of India ink and 1 ml. of croton oil in cavity). There is a pronounced infiltration with polymorphonuclear leukocytes, which reaches across the dermal muscle layer into the subcutaneous fat. Immediately beneath the ink-stained surface the connective tissue has undergone degeneration and contains few intact nuclei.

FIG. 3.—Another rat from Group II of the first experimental series (India ink, 0·2 ml., and croton oil, 1 ml., in cavity). This is a more advanced stage of the reaction type illustrated by the previous picture. The regions which, in Fig. 2, are infiltrated with leukocytes, have become devoid of cells (presumably due to disintegration of damaged leukocytes) and both muscle cells and connective tissue have undergone intense degeneration, with necrosis. In less affected regions of the same pouch, the polymorphonuclear leukocytes, or at least their fragmented bodies, were still discernible.

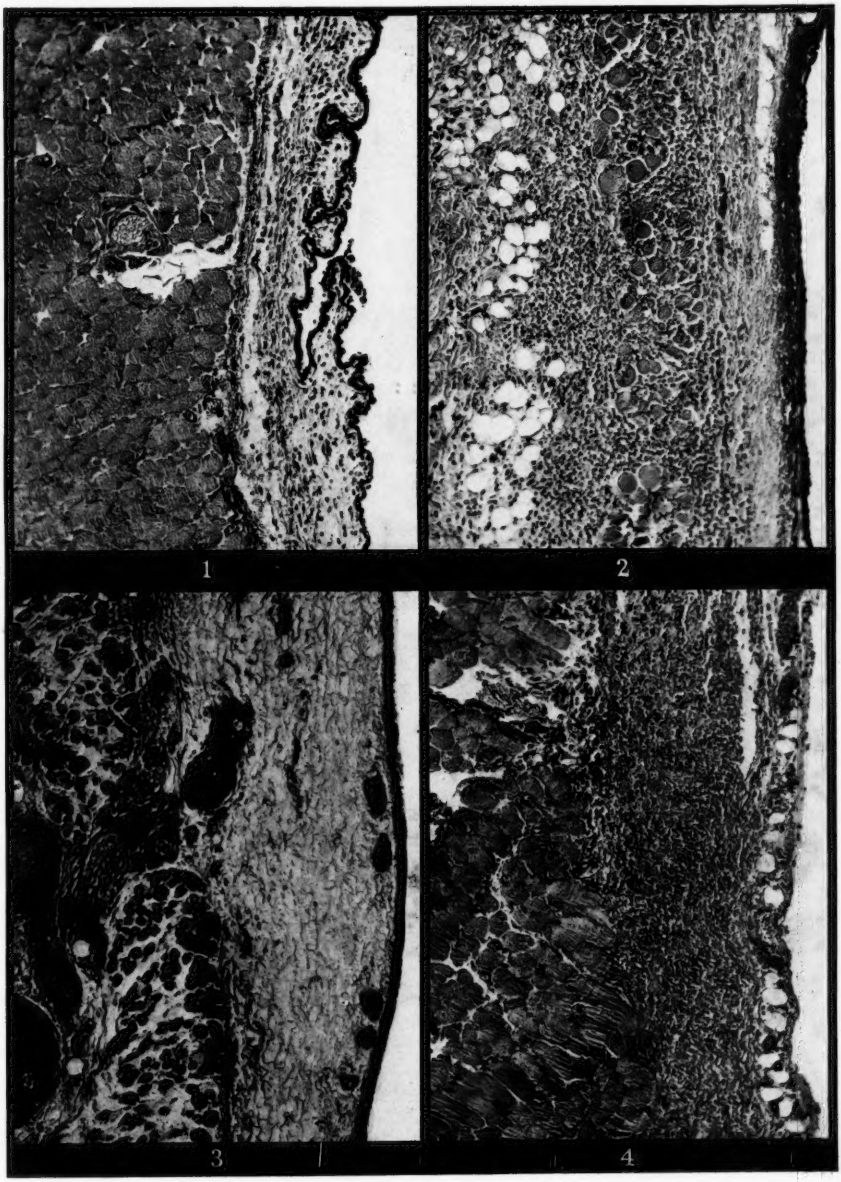
FIG. 4.—Rat from Group III of the first experimental series (NaCl solution, 2 ml., and croton oil, 1 ml., in cavity). Intense proliferation of fibroblasts and connective tissue fibres, arranged around newly formed capillaries. No necrosis or suppuration.

FIG. 5.—Rat from Group I of the second experimental series (India ink, 0·2 ml., in wall). The ink-stained inner surface of the pouch and, to a lesser extent, the dermal muscle, are heavily infiltrated with polymorphonuclear leukocytes. However, these infiltrates show no tendency to undergo liquefaction necrosis. They were not accompanied by skin necrosis in any animal.

FIG. 6.—Rat from Group II of the second experimental series (ink, 0·2 ml., in wall and croton oil, 1 ml., in cavity). In contrast with Fig. 5, the tissues near the inner margin of the pouch are almost devoid of cells and exhibit extensive liquefaction necrosis.

FIG. 7.—The progressive margin of the lesion shown in Fig. 6. The upper right part of the field resembles that illustrated in Fig. 6, but the lower left part still contains numerous well preserved polymorphonuclear leukocytes. Characteristically, the part most intensely stained with India ink is also the first to undergo necrosis due to croton oil.

FIG. 8.—Higher magnification of two fields from the upper and lower parts of Fig. 7, respectively. Note that the cells in the upper field which are heavily laden with carbon particles, are undergoing liquefaction necrosis and are separated from each other by fluid. In the lower part, the polymorphonuclear cells and histiocytes are clearly visible, since they took up virtually no carbon particles; here, the solid structure of the pyogenic membrane is still maintained, although a few leukocyte nuclei are beginning to disintegrate.



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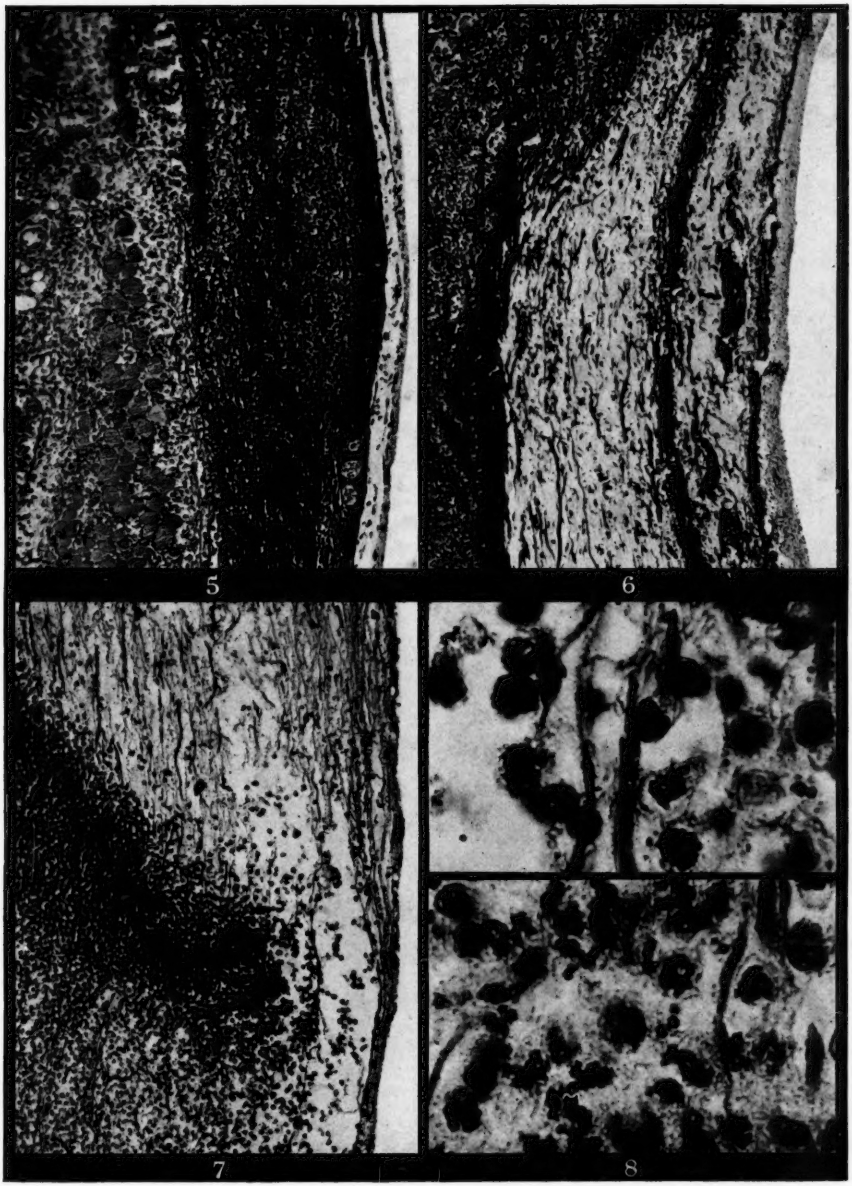

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the forceful distension of the connective tissue fibres when fluid is forced between them. In any event, this type of lesion does not tend to spread and has never led to necrosis of the skin in our experiments (Fig. 5).

On the other hand, if, after such an injection of India ink into the wall of the granuloma pouch, croton oil is introduced into the cavity, there is much suppuration. Later the polymorphonuclear leukocytes are destroyed, and the adjacent tissue becomes necrotic (Fig. 6). Hence, the combined effect of treatment with India ink and croton oil is essentially the same here as it was when both agents were introduced into the cavity (Fig. 2). The difference is merely that, when only a very small region of the wall is pre-treated with India ink, the subsequent introduction of croton oil into the cavity damages this pre-treated spot selectively.

In all these experiments, those polymorphonuclear cells which were laden with carbon particles were the most sensitive to the destructive effect of croton oil. Others, which were apparently attracted to this region after all the free carbon particles had been ingested, contained no ink granules and these appeared much more resistant (Fig. 7 and 8).

DISCUSSION.

In all these experiments, only two irritants (India ink and croton oil) were used, in various combinations. Hence, it would be hazardous to make any generalisations concerning the particular rôle of a blockade of the reticulo-endothelial system in inflammation. It is conceivable that, if another material were used to saturate the phagocyte system, tissue resistance to croton oil would not be similarly diminished. We must also keep in mind that pre-treatment with croton oil tends to diminish tissue resistance to subsequent irritation by formaldehyde (Selye, 1953*b*) and here there is no proof that an involvement of the phagocyte system is of particular significance.

As has been pointed out elsewhere (Selye, 1953*a*), the morphological and biochemical manifestations of inflammation are essentially the same during the first few hours, no matter how tissue is irritated. This first non-specific phase is soon followed, however, by highly specific tissue responses, the nature of which depends on the irritant used. For instance, the large cellulose particles contained in mustard powder stimulate giant-cell formation most prominently, kaolin (like India ink) results in a great abundance of macrophages, turpentine produces frankly suppurative tissue reactions, while croton oil stimulates the proliferation of fibroblasts more intensely than that of any other cell type. It is highly probable that these inflammatory reactions are adapted to the requirements of optimal defence against each type of irritant, and that differentiation in one direction diminishes adaptability in another sense. Thus, in the present experimental series, we may well imagine that if most of the cells in a certain region are " occupied " by the requirements of phagocytosis, their ability to undertake other local defensive functions is commensurately diminished. In other words, adaptation to one type of local defensive function diminishes capacity for another.

On the other hand, if a certain type of tissue response is elicited by one agent, the resulting structural and functional adjustments actually increase tissue resistance to a subsequently administered second dose of the same agent or one which requires a similar adaptive response. This is in agreement with our earlier observations showing, for instance, that pre-treatment with croton oil, which evokes a strongly fibroblastic response, actually increases resistance to a variety of other

substances (*e.g.*, trypsin, pepsin, bile, NaCl, NaOH), all of which induce similar tissue responses.

There is a striking similarity between the effect on topical irritation of India ink on the one hand, and antiphlogistic hormones (ACTH, hydrocortisone, cortisone) on the other. As we have shown elsewhere, the latter also predispose to necrosis, while they inhibit a normal inflammatory response to injury (Selye, 1953*d*). In fact, it has been assumed that the necrosis-enhancing effect of antiphlogistic hormones may be the direct consequence of their inhibitory effect upon inflammation. Presumably, in the absence of a well-formed granulomatous barricade, the irritant can more readily penetrate and hence damage adjacent tissues. India ink pre-treatment does not inhibit inflammation, but orients it in the direction of phagocytosis. The end-result, however, is the same, because the cells so affected cannot readily be transformed into a protective fibrous granuloma.

In other words, both the antiphlogistic hormones and the phagocytosis-promoting agents presumably enhance necrosis because they inhibit an appropriate inflammatory response to subsequently applied irritants. In the former case, this is due to a general inhibition of cell reactivity ; in the latter, to a diversion of mesenchymal activity in the direction of phagocytosis, which prevents it from optimally functioning for other topical defensive purposes.

It is desirable to obtain a better understanding of the mechanism responsible for the morphogenesis of the various specific tissue reactions characteristic of individual diseases. Hence we are now using the granuloma pouch technique in an effort to duplicate these, under standard conditions, with various chemically identifiable pure irritants, alone and in combination.

SUMMARY.

Using the " granuloma pouch " technique, it was shown, in the rat, that pre-treatment of a well delimited connective tissue area with India ink changes topical resistance in such a manner that subsequent treatment of the same area with otherwise well tolerated doses of croton oil causes marked suppuration and necrosis.

Given alone, neither India ink nor croton oil induced such tissue damage, even when administered in larger amounts.

It is concluded that tissues exposed to both these irritants in rapid succession break down, because they cannot adjust themselves simultaneously to two agents, which must be met by essentially different adaptive tissue reactions.

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GLYCOLYSIS BY MUSCLE EXTRACTS IN LOCAL TETANUS.

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ALTHOUGH opinions on the site of attack of tetanus toxin are still divided, most of the experimental evidence suggests that the muscle contracture in local tetanus is caused by peripheral disturbances. Virtually nothing is known, however, of the exact biochemical mechanism which, consequent on the action of the toxin, leads to the extreme muscular rigidity of local tetanus. Information on carbohydrate metabolism of tetanus-intoxicated animals has been limited to incidental data on glycogen (Davenport, Davenport and Ranson, 1929; Wertheimer, 1929; Staudinger, 1946), lactic acid (Davenport *et al.*, 1928; Muntz, 1949), muscle phosphates (Davenport *et al.*, 1930) and adenosine triphosphatase (ATP-ase) activity (Muntz, 1949). No significant changes were reported except for a slight fall of the creatine phosphate content of muscle and a nearly complete disappearance of glycogen from muscle and liver in generalized tetanus. In previous communications experiments have been reported on the phosphate and glycogen content of rat muscle in local tetanus (Wensinck, 1951, 1952; Wensinck and Cohen, 1953). In addition data were given on the glycolysis by diaphragm and also by rat muscle extracts *in vitro*. A serious fall in the rate of glycogen synthesis *in vivo* and in that of glycolysis by diaphragm and by muscle extracts *in vitro* could be demonstrated. It was concluded that intoxication by tetanus toxin causes serious disturbances of carbohydrate metabolism. In the present paper more detailed information will be presented on the glycolysis by extracts from rat muscle in local tetanus.

MATERIALS AND METHODS.

Production of local tetanus.

Male albino rats, weighing 100–150 g. and starved for 48 hr. were used. Local tetanus was induced by intramuscular injection of toxin. The toxins were prepared by precipitation from toxic filtrates by saturation with $(NH_4)_2\ SO_4$ or addition of methanol (2/3 vol. of the filtrates) at $-10°$. No purification of the crude toxin was attempted. Signs of local tetanus developed about 24 hr. after the injection of 1 μg. of toxin into the muscles of the left hind leg. The muscles of the right hind leg remained unaffected and were used as controls.

Muscle extracts.

Muscle extracts were usually prepared 48 hr. after the injection of the toxin. The animals were killed and bled by decapitation. Weighed portions of muscle from the left and right hind leg were chopped with scissors and ground in a mortar with sand and 4 vol. of 0·067 M phosphate buffer (pH 7·4). The opalescent supernatant obtained by centrifugation (3000 r.p.m.) was used. Mortars, tubes and buffer-solutions were cooled to 4°. The nitrogen content of the extracts was estimated by the Kjeldahl method.

Glycolysis.

The incubation mixture consisted of muscle extract (1 ml.), 0·067 M phosphate buffer pH 7·4 (0·8 ml.), 0·1 M $MgCl_2$ (0·2 ml.) and substrate. The final volume was 3 ml. Samples were analysed before and after an incubation period of 30 or 60 min. at 37° for glycogen (Good, Kramer and Somogyi, 1933), glucose-1-phosphate (7 min. hydrolysis phosphate), fructose (Roe, 1934), lactic acid (Long, 1946), pyruvic acid (Friedemann and Haugen, 1942) and inorganic phosphate (Fiske and SubbaRow, 1925). Proteins were precipitated by the addition of 3 ml. 20 per cent (w/v) trichloroacetic acid (TCA). Barium-soluble and insoluble fractions of the TCA extracts were prepared according to Umbreit, Burris and Stauffer (1951); in order to obtain a quantitative precipitation of fructosediphosphate, ethanol was added to a final concentration of 10 per cent. Triosephosphate was estimated as inorganic phosphate formed in N NaOH at room temperature in a period of 20 min. In some experiments, triosephosphate was trapped by adding 0·1 M KCN pH 7·4 (1 ml.) to the incubation mixture. All figures in the Tables and Figs. have been corrected for blank values found when no substrates were added to the muscle extract.

Substrates.

Glycogen, glucose-1-phosphate, fructosediphosphate and 3-phosphoglyceric acid were used as alternate substrates. Glycogen was prepared from the liver of rabbits (Bell and Young, 1934). Glucose-1-phosphate was prepared from starch according to Sumner and Somers (1944); the purity of the preparations was about 85 per cent. The barium salts of fructosediphosphate (Consolidated Midland Corporation) and 3-phosphoglyceric acid (Nutritional Biochemical Corporation) were converted to the sodium salts before use.

EXPERIMENTAL RESULTS.

It has been shown previously that the ability to convert glycogen into lactic acid is impaired in extracts prepared from rat muscle in local tetanus (t.m. extracts). These results could be confirmed in the present series. These experiments were extended to find the exact location of the blocking in the glycolytic chain. For this purpose various links in this chain were analysed separately *viz.*, the formation of lactic acid and pyruvic acid from glucose-1-phosphate, the formation of fructosediphosphate from glucose-1-phosphate, the formation of triosephosphate from fructosediphosphate and the formation of pyruvic acid from 3-phosphoglyceric acid. It will be shown that a compound, giving the Roe reaction for fructose, could be isolated from the Ba-insoluble fraction of incubation mixtures prepared from t.m. extracts. It was identified as fructosediphosphate. Since the aldolase activity of these extracts and their ability to convert 3-phosphoglyceric acid into pyruvic acid were found to be normal, it would seem that the accumulation of fructosediphosphate is due to inhibition of the complex enzyme system which is usually referred to as triosephosphatedehydrogenase.

TABLE I.—*Glycolytic Activity of Extracts from Normal and Tetanized Muscles.*

Substrates: glycogen, glucose-1-phosphate. Figures between brackets give initial amounts of substrates. Results are expressed as mg. found after incubation (60 min., 37°).

	Normal.	Tetanus.
Glycogen (10 mg.):		
Glycogen	1·9 ± 0·28	1·9 ± 0·48
Lactic acid	2·57 ± 0·114	1·35 ± 0·112
Glucose-1-phosphate (8·5 mg.):		
Glucose-1-phosphate	<1·0	<1·0
Lactic acid	3·18 ± 0·077	1·64 ± 0·148

Formation of lactic and pyruvic acid from glycogen and glucose-1-phosphate.—As shown in Table I, glycolysis is inhibited in t.m. extracts ; about half the normal amount of lactic acid is formed from both glycogen and glucose-1-phosphate. In t.m. extracts, lactic acid formation comes to an end rather abruptly after an incubation period of about 15 min. (Fig. 1). It will be seen from Table I that no differences in residual glycogen or glucose-1-phosphate were found between normal and t.m. extracts. Decreased lactic acid formation cannot be attributed, therefore, to an inhibition of phosphorylase or phosphoglucomutase activity. The formation of pyruvic acid from glucose-1-phosphate is also diminished in t.m. extracts (Fig. 1). Presumably, the blocking of glycolysis is located between the glucose–6–phosphate and the pyruvic acid stages. Pyruvic plus lactic acid formed from 8·5 mg. glucose-1-phosphate amounts to 3·55 mg. in normal extracts ; in t.m. extracts, about 1·75 mg. of these products are formed.

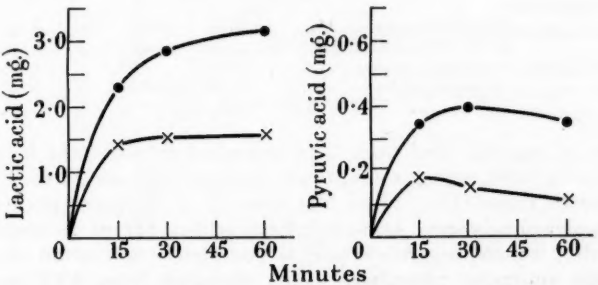

FIG. 1.—Formation of lactic and pyruvic acid during incubation of extracts from normal (.——.) and tetanized (x —— x) muscles. Substrate : 8·5 mg. glucose-1-phosphate.

Formation of fructosediphosphate from glucose-1-phosphate.—Virtually no compounds giving a fructose reaction are found in normal muscle extracts after incubation with glucose-1-phosphate under the conditions described for Table I. In t.m. extracts, on the other hand, such a compound accumulates. The amount of it found in these extracts (equivalent to $1·39 \pm 0·62$ mg. of fructose) accounts largely for the difference in lactic plus pyruvic acid formation (1·80 mg.) between normal and t.m. extracts. More than 95 per cent of the compound concerned was recovered in the Ba-insoluble fraction of the TCA extracts of the incubation mixtures. About 0·15 mg. of its total P content of 0·35 mg. was liberated on acid hydrolysis at 100° as inorganic P within 7 min. ; the remainder was hydrolyzed within 180 min. These properties suggest that the compound is fructosediphosphate and that it accumulates in t.m. extracts as a result of a blocking in the glycolytic chain.

Formation of triosephosphate from fructosediphosphate.—It may be concluded from the results shown in Table II that considerably less fructosediphosphate is broken down by t.m. than by normal extracts. This finding is in harmony with the accumulation of fructosediphosphate which occurs during incubation of t.m. extracts with glucose–1–phosphate. In the presence of cyanide, however, which traps triosephosphate, about 90 per cent of the fructosediphosphate added disappears in both normal and t.m. extracts. Thus the enzymic activity of aldolase is not altered in t.m. extracts. Significantly more triosephosphate is trapped

by cyanide in t.m. than in normal extracts. This phenomenon may be explained by assuming that in normal extracts the trapping by cyanide is only partial. Much triosephosphate escapes the trap and reacts with the active triosephosphate-dehydrogenase present which competes with the cyanide. It is then further broken down. In the case of the t.m. extracts more of the triosephosphate formed is caught in the cyanide trap because there is less triosephosphatedehydrogenase present to compete.

TABLE II.—*Formation of Triosephosphate during Incubation of Extracts from Normal and Tetanized Muscles with Fructosediphosphate.*

Substrate: fructosediphosphate (4·2 mg. fructose). Results are expressed as mg. fructose and mg. alkali-labile phosphate-P found after incubation (30 min., 37°).

	Normal.	Tetanus.
Fructosediphosphate:		
Fructosediphosphate (without cyanide)	0·63 ± 0·082	2·26 ± 0·171
,, (cyanide added)	0·36 ± 0·032	0·46 ± 0·042
Alkali-labile P (without cyanide)	0·09	0·24
,, (cyanide added)	0·66 ± 0·086	1·02 ± 0·035

Formation of pyruvic acid from 3-phosphoglyceric acid.—On incubation with 3-phosphoglyceric acid, equal amounts of pyruvic acid are formed by t.m. and normal extracts (Table III). Since the amount of inorganic phosphate formed equals the 3-phosphoglyceric acid-phosphate added before incubation, it must be assumed that 3-phosphoglyceric acid is completely converted to pyruvic and lactic acid, the inorganic phosphate being liberated from ATP by the muscle ATP-ase. The lactic acid formation was not determined in this series.

The results indicate that the conversion of 3-phosphoglyceric acid into pyruvic and lactic acid proceeds normally in t.m. extracts.

TABLE III.—*Formation of Pyruvic Acid and Inorganic Phosphate during Incubation of Extracts from Normal and Tetanized Muscles with 3-Phosphoglyceric Acid.*

Substrate: 3-phosphoglyceric acid (0·76 mg. P). Results are expressed as mg. pyruvic acid and mg. inorganic-P found after incubation (30 min., 37°).

	Normal.	Tetanus.
3-phosphoglyceric acid:		
Pyruvic acid	1·47 ± 0·025	1·42 ± 0·023
Inorganic phosphate	0·76 ± 0·062	0·75 ± 0·047

Localization of the site of inhibition of glycolysis in t.m. extracts.—The results mentioned in the preceding sections indicate that the blocking of glycolysis in t.m. extracts must be located between the triosephosphate and 3-phosphoglyceric acid stage; presumably the triosephosphatedehydrogenase system is inhibited.

It is obvious that this type of blocking must lead to an accumulation of fructose-diphosphate as the equilibrium constant of the aldolase reaction favours formation of fructosediphosphate.

Protein content of muscle extracts.—It seems possible that the extraction of enzymes from muscle might be influenced by the highly abnormal condition of

local tetanus. No differences in total nitrogen content were found between normal and t.m. extracts (mg. N/ml. : normal extracts 2·90 ± 0·046, t.m. extracts 2·93 ± 0·063). Differences in the nature of the protein, however, do exist. They are under investigation at present.

Relationship between onset of local tetanus and inhibition of glycolysis.—Under the experimental conditions used, local tetanus develops after an asymptomatic period of about 24 hr. Forty eight hours after the injection of toxin the treated hind leg is held in a stretched position as a result of an unrelenting muscular contracture. Undoubtedly, the blood circulation in the affected muscles will be influenced by this condition and it might be suggested that the observed inhibition of glycolysis is a non-essential phenomenon brought about by abnormal circulation. In order to obtain more information on the relation between the blocking of

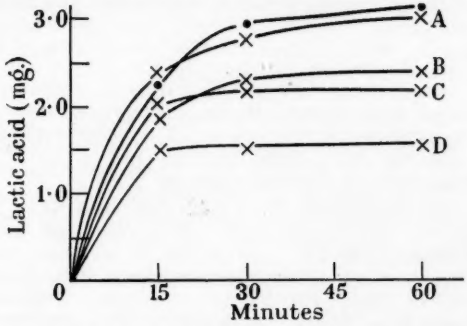

FIG. 2.—Glycolytic activity of extracts of normal (.——.) and tetanized (x —— x) muscles. Substrate : 8·5 mg. glucose-1-phosphate. Extracts were prepared 20 (A), 24 (B), 28 (C) and 48 (D) hr. after injection of toxin.

glycolysis and local tetanus, the glycolytic activity of muscle extracts was determined at the end of the asymptomatic period (20 and 24 hr. after the injection of toxin) and in an early stage of local tetanus (28 hr. after the injection of toxin). The results show that progressive inhibition of glycolysis is already noticeable before impairment of muscular function becomes obvious (Fig. 2). Since gross changes of the circulation are unlikely to occur at that time it would appear that inhibition of glycolysis is an early symptom of local tetanus, independent of changes in the circulation.

DISCUSSION.

It is shown that the glycolysis by extracts prepared from muscles in local tetanus is inhibited. This inhibition is due to a decrease in activity of the triosephosphatedehydrogenase system. It is an early symptom of local tetanus and seems to be independent of changes in the blood circulation. Since the glycolysis of intact diaphragms from animals suffering from a generalized tetanus is also inhibited (*cf.*, Wensinck and Cohen, 1953) it would appear that the inhibition of glycolysis in muscle extracts is not an artificial condition caused by changes in the extraction of glycolytic enzymes. Moreover the protein content of normal and t.m. extracts is the same. It has been stated (Wensinck and Cohen, 1953) that the glycolytic activity of t.m. extracts might be completely restored by the

addition of coenzyme I. This fact suggests that the inhibition of the triosephosphatedehydrogenase system may be due to suboptimal availability of coenzyme I. This supposition has gained more interest by evidence, to be presented in experiments by Wensinck and Boevé, not yet published, in which an acceleration was found of the degradation of coenzyme I in t.m. extracts.

SUMMARY.

The glycolytic activity of extracts prepared from rat muscles in local tetanus is inhibited.

This inhibition occurs in an early stage of the development of local tetanus and is accompanied by an accumulation of fructosediphosphate.

The inhibition of the glycolysis is due to a deficient function of the triosephosphatedehydrogenase system.

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SURVEY OF PAPERS.

BANGHAM, MAGEE and OSBORN have studied the removal of particles from the peritoneal cavity in rats and mice. The particles used were of glass containing radioactive caesium, the distribution of which could therefore be determined with a Geiger counter either in excised lymph nodes or in the intact animal. The effect of posture on drainage was confirmed. Angular particles, which provoked an inflammatory response, were removed more rapidly than spherical, and mild degrees of inflammation otherwise induced caused no interference with removal at first, although some fixation occurred later (p. 1).

MOLLIE BARR and MONA LLEWELLYN-JONES criticize the evidence in favour of immunisation with two antigens simultaneously as inadequate, and have re-examined the effects of this proceeding in animals. Combinations both of two toxoids and of a toxoid with a vaccine were tested, and the response was found to vary with the total dose and with the relative proportions of the two components. When there was some pre-existing immunity to one component, there was a preferential response to this component and an inadequate one to the other. Since either synergism or interference may result according to the conditions, the general adoption of such proceedings without further study is undesirable (p. 12).

MANTEN and ROWLEY have failed to confirm Voureka's observation that treatment with chloramphenicol and antiserum induces profound changes in *Bact. coli*. They found that either antiserum or normal serum acted in the presence of chloramphenicol simply by prolonging survival and thus enabling certain variants to develop (p. 23).

HILL and CRUICKSHANK prepared antisera in rabbits to whole kidney, renal glomeruli and lung from rats. These sera conjugated with fluorescein were applied to frozen sections of rat kidney and the cells to which antibody became attached were identified by fluorescence microscopy. From the results obtained with different sera it is concluded that kidney contains distinct antigens, in basement membrane and tubular epithelium respectively. Antiserum to the former injected intravenously produces nephritis. It is suggested that the use of an antibody-fluorescein combination as a histochemical reagent may prove useful in other directions (p. 27).

GOWANS has carried out experiments designed to determine whether antibiotics, and in particular the newer antibiotics credited only with a bacteriostatic action, can actually destroy bacteria *in vivo* and thus eliminate infection without co-operation by leucocytes. Rabbits, in some of which extreme granulocytopenia had been produced by injections of benzene, were inoculated intradermally with pneumococci and treated with penicillin or terramycin; the progress of the infection was studied in films and cultures of the local lesion and by blood cultures. Even in the absence of phagocytosis, the cocci became Gram-negative and disintegrated and the lesions became sterile. It is concluded that both antibiotics must exert a direct bactericidal action (p. 35).

MITCHELL found that of 14 sera from patients with molluscum contagiosum, only 3 fixed complement with an antigen prepared from lesions of this disease. In one patient complement-fixing antibody was formed in response to immunisation with virus from his own lesions. This and a naturally positive serum did not fix complement in the presence of vaccinia, cowpox or fowlpox antigens. Various attempts to propagate molluscum virus in eggs were unsuccessful (p. 44).

GRANT has examined the effects of some mono- and polyhydric alcohols on precipitin reactions, this work having been suggested by the observation that ethanol increases the amount of precipitate formed in the zone of antigen excess. Although the results differed somewhat among the four precipitin reactions studied, in general ethanol and methanol accelerated the development of turbidity, which also reached a higher maximum, an effect attributable to the lower solubility of proteins in the presence of alcohol. Ethylene glycol and glycerol had an inhibitory effect, probably owing to their combination with hydrogen bonding sites on antigen or antibody molecules (p. 50).

POPE and MURIEL F. STEVENS have removed antibodies other than specific antitoxin from diphtheria antitoxin by absorption with toxin-free filtrates of *C. diphtheriae* culture, ground suspensions of *C. diphtheriae*, etc. The effects were observed by accurate determinations of optical density due to flocculation in mixtures of the antitoxin with toxin in different proportions. Whereas crude antitoxin gives a broad and irregular flocculation curve, a purified product gave one which was narrow and symmetrical. This product had an activity of 211,000 units per g. protein, which is higher than any hitherto recorded, but it is said to be still far from pure (p. 56).

WHITELEY, STONER and THRELFALL have compared the uptake of radioactive phosphorus in the skin of the lateral zones of rapid hair growth in rabbits with that in zones of slower growth. Uptake was considerably greater in the former, as determined after both short and long intervals; several factors are believed to operate successively in producing this difference (p. 73).

WUNDERLY, ELIZABETH GLOOR and HÄSSIG have submitted normal and pathological human sera to electrophoresis, cut off sections of the strips containing different proteins, extracted them, brought the extracts to constant protein concentration, and obtained precipitates from them, the optical density of which was measured, by admixture with rabbit anti-human serum. The pathological sera showed marked variations in content of different proteins, but no evidence was obtained that they included abnormal proteins. The density of these precipitates may have been affected by the presence of substances other than protein in the isolated fractions, and in this connection the results of staining electrophoretic strips for lipoids are described (p. 81).

SAPEIKA administered dicoumarol in the diet to rats and guinea-pigs in such doses as usually to cause haemorrhages, and found that the ascorbic acid content of the liver and adrenal was reduced. On the other hand, the simultaneous administration of ascorbic acid did not prevent these haemorrhages. For this and other reasons it is concluded that haemorrhage due to dicoumarol overdosage does not result from ascorbic acid deficiency (p. 88).

MORGAN and WINIFRED M. WATKINS have extended the study of agglutination of human red cells by extracts of plant seeds, using the seeds of six species and testing them with red cells of six groups. They have further studied the inhibition of this effect by blood group substances and other biological materials and by sugars, a number of which inhibit some of these agglutinins of plant origin but not others. The specificity of action of some sugars, shown to depend on slight differences in molecular structure, in relation to plant agglutinins acting mainly on either O or A and B cells, promises to yield information about the reactions concerned (p. 94).

FEINBERG and MORGAN have obtained from *Trichomonas foetus* (var. Manley) by extraction with diethylene glycol a specific substance reacting with antiserum to this organism and eliciting a skin reaction in infected or immunised animals. It reacted little with antiserum to *T. foetus* (var. Belfast). The substance has been extensively fractionated and shown to consist of a polysaccharide including at least five elements, and ten amino-acids. It does not itself induce antibody formation and may therefore be a hapten. A second, antigenically inert, substance isolated from *T. foetus* is a polysaccharide resembling glycogen (p. 104).

SURVEY OF PAPERS.

ALBERT, GIBSON and RUBBO in a continuation of previous work have examined the bactericidal action of 8-hydroxyquinoline (oxine). Using *Staph. aureus* they found that oxine had no action in the absence of traces of copper or iron, that dilute solutions were more active than stronger ones, and that the action depended on a balanced ratio between oxine and iron. A hypothesis to account for these findings is put forward (p. 119).

BIRD absorbed 32 Group 0 sera with A- and B-specific substance, and found that in many of them not only was all agglutinin for the corresponding red cells removed, but some of that for cells of the other group. This was usually a one-way and less often a reciprocal effect, and varied in degree. There is some evidence that this " linkage " occurs not only in iso- but in auto-agglutinins (p. 131).

BARBARA RAYNER, MARGARET TUTT and JANET VAUGHAN have studied the distribution of yttrium in rabbit bones after intravenous injection by the same methods as they used in previous experiments with strontium. The findings were similar, but a higher proportion of yttrium was retained and persisted longer, and retention was less affected by the animal's age (p. 138).

DONIACH and LOGOTHETOPOULOS have tested the goitrogenic action of various resorcinol preparations in rats, and produced thyroid hyperplasia by injections of resorcinol in oil or of resorcinol diacetate. The failure of previous workers using aqueous resorcinol is shown to be due to its transient effect (p. 146).

SAMPAIO and ISAACS find that heating normal rabbit serum with crude *V. cholerae* extract or with crystalline trypsin inactivates the Chu inhibitor which interferes with haemagglutination by freshly isolated strains of influenza A virus. The Francis inhibitor, much more active against heated influenza B virus, was greatly reduced after heating with trypsin in most of the animal sera tested. Specific antibody to PR8 virus was unaffected by this treatment (p. 152).

BURTON has studied the distribution of phosphatase in the endometrium throughout the menstrual cycle by a modified Gomori's method using undeparaffinised sections, which was less prone to artefact production than the original technique. After accumulation at the free border of the cells the phosphatase was discharged into the glandular lumen soon after ovulation, and its persistence there leads the author to suggest a function of the enzyme additional to that concerned in endometrial development (p. 159).

EVA S. ORLANS has studied the effect of photo-oxidation on four antibody-containing sera, and finds that after this treatment a progressive loss of reactivity occurs, which is attributed to the formation of incomplete antibody. The effects of adding antigen to mixtures of such serum and untreated serum were also studied. It was also shown that photo-oxidation reduces the tryptophane content of antibody globulin (p. 164).

HOWARD, WALLACE and PAYLING WRIGHT have studied the capacity of various sterols to inhibit haemolysis by streptolysin O, and defined the molecular structure on which this capacity depends. This inhibition is attributed to combination of streptolysin with the sterol instead of with cholesterol in the red cell envelope (p. 174).

HOWARD and WALLACE have tested the resistance of red cells of 17 animal species to streptolysin O and to saponin, and found those of the mouse to be exceptionally resistant to the former (p. 181).

HOWARD and WALLACE show that mice are also exceptionally resistant to the lethal action of streptolysin O. Its haemolytic and lethal action could not be dissociated by any of several treatments. Hypercholesterolaemia does not protect against this toxin despite its neutralizing effect *in vitro* evidently because the cholesterol is bound to plasma globulins (p. 185).

HOLMAN and MCLEOD criticize the use of Schales' reagent by Jordan in demonstrating H_2O_2 production by *P. septica* and define two possible sources of error when this reagent is so used (p. 191).

GOWANS has studied the effect of antibiotics on peritoneal infection with pyogenic cocci in normal and leucopenic mice by cytological as well as cultural examination of the exudate. Phagocytosis in normal mice contributed to the destruction of larger numbers of organisms, but in its absence many were destroyed by the drugs alone. In both groups the few surviving cocci eventually multiplied and caused death (p. 195).

VAN HEYNINGEN and GLADSTONE describe methods for extracting and purifying the neurotoxin of *Sh. shigae* and some of its properties. This product, the yield of which is small, is electrophoretically homogeneous, and its toxicity for the rabbit equals that of tetanus and botulinus toxins for the guinea-pig (p. 202).

BALDWIN, using only 4·5 mg. of the purified toxin described in the preceding paper, has determined its sedimentation coefficient and other properties in the Svedberg ultracentrifuge (p. 217).

VAN HEYNINGEN and GLADSTONE have studied the effect of the iron content of the medium on the production of neurotoxin by *Sh. shigae* and its relationship to growth and various metabolic activities, some of which are affected by iron content and others not. Toxin production diminishes with increasing iron content, although not in direct proportion (p. 221).

VAN HEYNINGEN and GLADSTONE describe and illustrate a semi-micro flocculation method whereby the toxin content of as small a volume as 0·2 ml. of fluid may be assayed (p. 230).

SURVEY OF PAPERS.

Mollie Barr and Mona Llewellyn-Jones have extended their studies of the response to two immunizing agents given together. The response to tetanus toxoid when combined with T.A.B. vaccine or diphtheria toxoid was diminished in animals which had had a previous injection of the other component only; on the other hand T.A.B. vaccine given between two doses of T.A.B. vaccine plus tetanus toxoid actually enhanced the response to the latter (p. 233).

Pope and Muriel F. Stevens have further purified diphtheria antitoxin by absorption of antibodies other than antitoxin itself, and obtained a product shown by test *in vivo* to contain considerably more antitoxin than that indicated by assay *in vitro*. The broad equivalence zone in the latter test (*i.e.*, range of proportions in the mixture in which free toxin or antitoxin cannot be detected in the supernatant after flocculation) was replaced by a sharp equivalence point (p. 241).

Pollock has sought an explanation of the fact that anaerobiosis lasting 100 minutes is followed by diminished penicillin-induced penicillinase formation by *B. cereus*, whereas after only 25 minutes it is at once resumed at the normal rate. Two processes are believed to operate in this effect, one of which also depends on the interval between adding penicillin and the establishment of anaerobiosis (p. 251).

Marrack and Grant have determined the minimum concentrations of salt permitting flocculation in mixtures of antigen and antibody, and shown that these vary with the nature of the latter and with pH. Evidence was obtained that antigen and antibody combine even in salt concentrations too low to permit flocculation (p. 263).

Gell and Isobel T. Hinde have shown by two methods that cortisone does not affect the phagocytic activity of cells of the reticulo-endothelial system (p. 273).

Harris has used a photomicrographic trace technique to study chemotaxis in rabbit monocytes. Except that they moved more slowly and possibly more directly, they were found to react in the same way as granulocytes, which had previously been examined by the same method (p. 276).

Weinbren has studied the effects of bile duct obstruction on rat liver regeneration by comparing the gain in weight of the liver residuum following partial hepatectomy with or without accompanying biliary obstruction. These results together with those of certain control experiments suggest that liver regeneration in biliary obstruction can take place provided the stimulus of liver cell loss is present (p. 280).

Brown has compared the complement-fixing, immunizing and skin-reactive properties of soluble and particulate fractions of herpes simplex virus. The soluble phase contained most of the complement-fixing activity and produced skin reactions in guinea-pigs hyper-immunized with living virus. Guinea-pigs injected repeatedly with this fraction developed skin sensitivity but complement-fixing antibody was not found in their sera. The washed virus fixed complement weakly. Sera made against formolized particles possessed neutralizing but not complement-fixing activity; the reverse of this was found in hyperimmune sera which had been absorbed with washed particles (p. 290).

LE BOUVIER has used the complement fixation method of Fulton and Dumbell to study reactions between a type 2 poliomyelitis antigen made from an aqueous suspension of mouse brain and sera from various hyperimmunized animals including monkeys receiving adjuvant mixtures. Homologous sera from these animals reacted specifically with type 2 virus; those made against types 1 and 3 did not. Low titre specific fixation was obtained with a few human sera of a small group of paired samples collected from cases of poliomyelitis (p. 300).

FRASER has studied the production of a recombinant strain (N-M) of influenza virus in 1st cycle fluids after double infection of the washed allantoic membrane with NWS and MEL strains introduced at different times. When the interval was more than about 2 hours N-M was not recovered and interference by NWS with the growth of MEL occurred. The production of N-M was associated with the appearance of haemagglutinin susceptible to antisera against both parent strains (p. 319).

DENZ has studied the distribution of cholinesterases at the myoneural junction in rats' diaphragms by histochemical and chemical methods. A comparison was made of the staining reactions using three methods with different substrates, and the localization of cholinesterases identified by the employment of selective inhibitors. Synaptic gutters were found to contain mainly specific cholinesterase though there was also some of the non-specific variety (p. 329).

SURVEY OF PAPERS.

FONNESU and SEVERI have studied the changes produced in the kidney by bacterial toxins, mercuric chloride and renal pedicle ligation by histochemical and chemical methods. Bacterial toxins produced cloudy swelling and diminution in DNA content, while mercuric chloride and renal pedicle ligation produced necrotic areas in which both DNA and RNA were reduced. They suggest that true cloudy swelling may only be caused by bacterial toxins and that mercuric chloride and renal pedicle ligation produce more severe degenerative changes. They have also shown that the water content in cloudy swelling is not increased (p. 341).

STAMP has sought an explanation for the varying immunizing activity of *Str. pyogenes* vaccines, with particular regard to the possibility that proteinase formed in the culture may reduce this activity by action on the "M" protein substance. That this is a true explanation is supported by observations of three kinds; that strongly proteinase-producing strains make poor vaccines; that weak producers make poorer vaccines under cultural conditions (pH, O/R potential) favouring proteinase formation, and that proteinase added to a culture forming little or none of it reduces the immunizing capacity of the vaccine so derived (p. 347).

WHITELEY, STONER and THRELFALL have shown that shock depresses mitosis and the uptake of phosphorus in the hair follicles of rabbit skin, and relate this effect to changes in the skin circulation. The assumption made by other workers that an indirect or hormonal mechanism may be responsible is unnecessary to explain the changes observed (p. 365).

FRANCES D. BOSANQUET, DANIEL and VOLLUM have investigated the response of the leptomeninges to tuberculoprotein in normal and sensitised guinea-pigs. They have shown that a tuberculin reaction occurs which is analogous to that produced in the sensitised animal in the skin, and the relationship of this reaction in guinea-pigs to tuberculous meningitis in humans is discussed (p. 376).

JENIFER JOWSEY, BARBARA RAYNER, MARGARET TUTT and JANET VAUGHAN, using an autoradiographic technique, studied the localisation of intravenous ^{90}Sr in the bones of rabbits. In both young (1 day old) and older animals (5–7 weeks) concentration occurred in areas of active growth, though in the younger group much subsequent diffuse redistribution took place. In the older group the possible effect of persistent localisation on tumour production is considered (p. 384).

MACNAMARA has shown by serological studies in monkeys and by cross-protection tests in mice that there is a slight and not strictly reciprocal cross-relationship between Uganda S and yellow fever viruses (p. 392).

FENNER and GWENDOLYN M. WOODROOFE report observations on infectious myxomatosis, the deliberately introduced disease which has caused a high mortality in wild Australian rabbits. They have studied the spread of the virus in the body, its concentration in the skin lesions (important because a mosquito is the vector), shown that virus in the blood is not free but associated with leucocytes, and that antibody appears in the late stages (p. 400).

MENKIN describes experiments which are interpreted as showing that cortisone penetrates an area of inflammation and there neutralizes the action of leukotoxine, an effect to which its beneficial action in certain conditions is attributed. In order to demonstrate this it is necessary also to administer ACTH to neutralize the action of exudin (p. 412).

MENKIN describes similar experiments to those reported in the foregoing paper, indicating that ACTH penetrates and accumulates in an inflamed area, thus preventing the increased capillary permeability due to exudin and to some extent inhibiting leucocytic diapedesis (p. 420).

HALPERN, BENACERRAF and BIOZZI, using intravenous injections of commercial India ink, have found that large doses increase the rate of blood clearance and cause a variation in distribution of carbon particles in organs of rats, rabbits and mice. These findings were attributed to a coagulation of fibrin *in vivo* and the theory is confirmed here by repeating the experiments with additional simultaneous injections of substances which modify blood coagulation (thromboplastin, thrombin, heparin, and dicoumarol). In further experiments with commercial India ink and a special ink containing no shellac it was found that large amounts of thromboplastin were liberated and the blood fibrinogen lowered as a result of the shellac present in the commercial preparation. The toxicity of this preparation to animals was also found to be due to the shellac content (p. 426).

BIOZZI, BENACERRAF and HALPERN have studied the rate of phagocytosis of carbon in the spleen and liver after intravenous injection of a suspension free from shellac by quantitative methods. The rate is increased in proportion to carbon concentration in the blood and diminished in proportion to the amount already phagocyted ; an equation to express this relationship is proposed. A relationship between the weight of the liver or spleen and its phagocytic activity has also been demonstrated (p. 441).

MAGEE and PALMER have studied the rate of drainage from the peritoneal cavity to the retrosternal lymph nodes in rats and mice by means of radioactive glass microspheres. A comparison of control and cortisone-treated groups showed no differences. Further experiments in mice showed that cortisone neither retards nor accelerates removal of India ink particles as had been found by other workers (p. 458).

AMOS has used suspensions of mouse leukaemia cells to immunise three strains of mice resistant to this disease, and so obtained serum which agglutinated a suspension of mouse leucocytes, a method for preparing which is described. Absorption with liver and comparison with red cell agglutination were carried out in order to determine the specificity of the antibody formed (p. 464).

SURVEY OF PAPERS

SMITH, KEPPIE and STANLEY describe a method of collecting large quantities of *B. anthracis* and its products from infected guinea-pigs. The method is applicable to other infections (p. 471).

SMITH, KEPPIE and STANLEY have compared the anthrax bacilli harvested from guinea-pigs with others grown in artificial medium, and found them to have larger capsules and to be quite resistant to phagocytosis. They differ markedly from bacilli grown *in vitro* by dissolving in ammonium carbonate solution. Two methods of preparing extracts of *B. anthracis* grown *in vivo* are described (p. 477).

KEPPIE, SMITH and PATRICIA HARRIS-SMITH have examined the intra- and extracellular products of *B. anthracis* described in the previous papers. Both were found to be non-toxic on intraperitoneal injection; both contained aggressins, but only extracellular products elicited active immunity. It is suggested that the extracellular aggressin and the protective antigen may be identical (p. 486).

BOWDEN has studied the cytology and bacteriology of pneumococcal infection in animals treated with aureomycin, and concludes that this drug must exert a direct and rapid bactericidal action (p. 497).

GHOSH RAY and SWAIN have studied the development of antibodies in 31 patients with mumps, using complement fixation with soluble (S) and virus (V) antigens, haemagglutinin-inhibition and neutralization in embryonated eggs. The early production and rapid decline of S antibody was in contrast with the appearance at 5–8 days of V antibody which reached a higher titre and persisted for very much longer; in sera collected 18 months later the amount was about four times that in the acute phase. Even higher antibody levels but similar patterns of formation followed by slow partial reduction were obtained by haemagglutination-inhibition and neutralization (p. 501).

WILSON SMITH, COHEN, BELYAVIN and WESTWOOD have investigated the effects of ether treatment on eluates of the two main types of influenza virus. Carefully standardized procedures were used to assess and compare the action on the specific complement-fixing, haemagglutinating and enzymic activities of the virus suspensions. It was in this order that inactivation of these properties proceeded and this is the reverse of the sequence found on heating. The haemagglutinating activity was altered by ether in such a way as to reduce the velocity of red cell aggregation. In titrations by the densitometer method this qualitative change in the virus affects the apparent titre independently of any quantitative reduction (p. 512).

COOMBS, HOWARD and MYNORS combined soluble protein antigen with red blood cells. When they added a non-precipitating antibody, they could detect the combination between antigen and antibody by agglutinating the antibody-antigen-red cell unit with antiglobulin specific for the species from which the antibody had been obtained (p. 525).

LOVELL, SCOTT, HUDSON and OSBORNE have noted delay of bruise dispersion in Cushing's syndrome and in cortisone- or ACTH-treated cases of other diseases. Similar delay was found experimentally in cortisone-treated guinea-pigs (Cushing). They suggest that delayed disappearance may be due to alteration in connective tissue ground substance or to impaired lymphatic removal. Diminished macrophage activity seemed unlikely to be an operative factor (p. 535).

CLARK, LATHE and RUTHVEN fractionated lipids from placenta and brain in order to determine which compounds were responsible for the inhibitory effect of lipid extracts of tissues on the agglutination of red blood cells by appropriate antisera. They found that the polar as opposed to the non-polar lipids were the anti-agglutinins (p. 542).

ANDERSON, seeking a method of identifying enterotoxin-producing strains of *Staph. pyogenes*, has used isolated segments of rabbit intestine. Toxic filtrates from 12 pyogenic strains were without effect, but filtrates from 12 food poisoning strains all produced a characteristic increase in tone (p. 548).

CHACKO has studied the Treponema Immobilization Test for syphilis, and confirmed in the rabbit that the antibody responsible is distinct from the Wassermann reagin. It is also very stable, surviving heat, storage, and bacterial contamination. The necessity for preparing treponemal suspensions only from very early testicular lesions in rabbits was also demonstrated (p. 556).

HOWARD has shown that the haemolysin of *Cl. histolyticum* is oxygen-labile, inhibited by cholesterol and neutralized by antistreptolysin O, properties which indicate a close relationship with several toxins formed by other species (p. 564).

BARNETT, BUSHBY and MITCHISON have shown that highly isoniazid-resistant strains of tubercle bacilli have a much diminished degree of virulence for guinea-pigs and mice, lesions even in the former being non-progressive, the bacilli dying out. Treatment of these infections with isoniazid had no effect in guinea-pigs ; in some experiments in mice the disease appeared to be aggravated (p. 568).

SURVEY OF PAPERS.

CRUICKSHANK and LOWBURY have studied the effect of pyocyanin on tissue cultures of human skin and on human leucocytes. The concentration of pyocyanin in green exudates from infected burns has been estimated, and found to be sufficient to damage skin *in vitro*, although rarely high enough to affect leucocytes (p. 583).

CZEKALOWSKI, MCLEOD and RODICAN have cultivated leptospira in solid and semi-solid media, and have confirmed Dinger's findings that discs of growth are formed just below the surface of the medium. Observations have also been made bearing on the respiratory mechanism of these organisms (p. 588).

MACKENZIE measured the excretion of urinary pepsinogen and found no difference between healthy subjects and those suffering from peptic ulceration. The enzyme was absent from the urine of 6 pernicious anaemia patients but was present in 5 others whose macrocytic anaemia was of a different type (p. 596).

HARRIS has studied the movements of lymphocytes by a photomicrographic trace technique previously used in a similar study of granulocytes and monocytes. No chemotactic reaction to various bacteria, cells parasitised with virus, and autolysed tissues, was observed in lymphocytes. Among other properties the author noted the inability of lymphocytes to adhere to glass, a useful distinction from monocytes (p. 599).

MACPHERSON, WILKINSON and SWAIN have shown that *Klebsiella aerogenes* and *Klebsiella cloacae* polysaccharides which have been treated with NaOH combine with red cells and inhibit their agglutination by viruses. The surface charge on these cells has been found to be increased, and a theory to explain the inhibitory action of polysaccharides is propounded (p. 603).

FISET and DONALD have shown that haemagglutinin-inhibiting antibody in the sera of ferrets immunized with influenza viruses can be readily absorbed by treatment with fowl red cells with which the homologous virus has been irreversibly united. For this purpose the virus is first altered to the indicator state. The average number of virus particles adsorbed on ghost cells could be calculated from counts on electron-micrographs. It was found that receptor-destroying enzyme (RDE) removed 30–50 per cent of the virus but subsequent exposure to antibody produced no further reduction. Fowl cells were used to adsorb different influenza viruses and, after RDE treatment, could be shown to absorb antibody in a strain-specific manner (p. 616).

WYNN WILLIAMS has found that intramuscular cortisone given to rats, rabbits, and guinea-pigs causes exophthalmos as estimated by measurement of the intercorneal distance. In a further experiment thyroidectomy was found to cause exophthalmos which was unaffected by cortisone treatment but diminished by desiccated thyroid. The possible mode of causation of exophthalmos is discussed (p. 621).

DEXTER, STONER and GREEN have found that periods of limb ischaemia in rats insufficient to produce severe shock are accompanied by an antidiuretic effect with excretion of an antidiuretic substance in the urine. They consider that the effect may in part be due to posterior pituitary hormone liberated by afferent impulses from the damaged limb (p. 625).

TOBIN has investigated the effects of varying doses of X-rays on bacteriophage in a lysogenic and in a sensitive strain of *Ps. pyocyanea* in different media. Induction of the temperate phage resulted and the optimum dose of X-rays and the proportion of bacteria in-

duced were determined. In the sensitive organism large doses interfered considerably with the power to initiate phage growth. The effects of X-rays were generally very similar to those observed by other workers using U.V. irradiation (p. 635).

SMITH and WOODIN injected aqueous extracts of ox cornea into rabbits and obtained precipitating antibodies. Some of the antigenic constituents of the extracts were also present in ox serum but two antigens appeared to be derived from the connective tissue of the ox cornea (p. 647).

BETTY REYNOLDS and ROWLEY have shown by the use of radio-active penicillin that *Str. faecalis* takes up no more penicillin when streptomycin is also present. The synergic action of these two antibiotics can therefore not be due to a greater permeability of the bacterial cell wall to penicillin produced by streptomycin (p. 651).

LINDER, PARKER and SELZER have performed tests of liver function in rats fed on diets tending to produce liver necrosis. Even in animals with little or no change in the liver there was marked bromsulphthalein retention, although the thymol turbidity test, the colloidal gold reaction and serum protein estimations usually gave normal results (p. 656).

JENIFER JOWSEY, MAUREEN OWEN and JANET VAUGHAN describe the findings in micro-radiographs and autoradiographs of a single tibia of each of two monkeys given a fatal dose of ^{90}Sr. This element was found to be concentrated in areas of bone growth, and to be distributed irregularly in " building sites," the highest concentrations in these sites being assumed to have occurred when osteoblastic activity predominated in them at the time of administration (p. 661).

WHITE has studied the activity of Taylor's 1233 virus in relation to the virus-receptors of human red cells. Treatment of the cells with varying dilutions of receptor-destroying enzyme (RDE) destroyed receptors for 1233 virus in such a way as to place it in the gradient series between WSE and LEE influenza strains. The agglutinin titre of 1233 virus was considerably reduced by treatment of the red cells with NDV or SW viruses, an effect not observed after treatment with LEE or MEL. The 1233 strain appears to act on a set of red cell receptors susceptible also to the action of RDE and, to some extent, to SW and NDV but different from those mainly responsible for haemagglutination by viruses of the influenza group as a whole (p. 668).

RINDANI and SELYE produced subcutaneous cavities in rats by injecting air, and showed that when croton oil was introduced into such a cavity after India ink it caused much more damage than the same or a larger dose of either material separately. They conclude that tissues cannot respond simultaneously to two different agents each eliciting a different type of reaction (p. 674).

WENSINCK, BOEVÉ and RENAUD prepared extracts from tetanic muscle and found that they were deficient in triosephosphatedehydrogenase activity. Less lactic acid was formed from added glycogen than in extracts from normal muscle and fructosediphosphate accumulated (p. 681).

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